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(54) Title: COMPOSITION AND METHODS FOR CREATING SYNGENEIC RECOMBINANT VIRUS-PRODUCING CELLS (57) Abstract Replication-defective viruses and means for intracellular replication thereof are described which are useful for gene therapy. Human cells can be changed into recombinant replication-defective virus particle-producing cells by the simultaneous delivery to those cells of two different nucleic acids: the first being a replication-defective viral genome, the second being a nucleic acid that complements the viral sequences deleted from the first nucleic acid so as to result in the production of new infective virus. The first nucleic acid can be delivered by the replication-defective virus itself or, as a nucleic acid that is not part of the virus. In a preferred embodiment, the replication-defective virus includes elements to maintain the two nucleic acids in combination during transduction.		

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COMPOSITION AND METHODS FOR CREATING SYNGENEIC RECOMBINANT VIRUS-PRODUCING CELLS

BACKGROUND OF THE INVENTION

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Field of the Invention

The present invention is in the general field of gene therapy, and is in particular in the area of engineering viral vectors for transduction of normal cells.

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Description of the Related Art

"Gene therapy" refers to the treatment of pathologic conditions by the addition of exogenous nucleic acids to appropriate cells within the organism. Nucleic acids must be added to the cell, or
15 transduced, so that they remain functional within the cell. For most gene therapy strategies, the new nucleic acids are designed to function as new genes, i.e., code for new messenger RNA that in turn codes for new protein. As originally conceived, gene therapy was directed towards monogenetic disorders like adenosine deaminase
20 deficiency and cystic fibrosis. It has become abundantly clear that gene therapy might also be helpful in polygenetic somatic disorders like cancer.

The rapid implementation of gene therapy in human trials has been made possible by the development of relatively efficient
25 means of adding new nucleic acids to cells, a process generally referred to as "gene transduction". The clinically applicable gene transduction methods fall into one of three categories: a) cationic lipids, (b) molecular conjugates, and (c) recombinant viruses. These different means of accomplishing gene transduction have been
30 reviewed.

Although the three major groups of gene transduction methodology are relatively efficient, the percentage of target cells that can be transduced *in vivo* remains relatively low. One pathophysiologic condition where transduction efficiency has been
35 shown to be a limiting factor is cancer. Some strategies for cancer gene therapy entail the addition of toxin genes, or other genes deleterious to cancer growth, to the tumor mass. These approaches have been facilitated by the use of toxin genes that kill not only the

transduced cells, but also adjacent cells by a "bystander effect". The herpes simplex virus thymidine kinase + ganciclovir system exemplifies the desired bystander effect. In this case, the viral thymidine kinase gene converts the prodrug, ganciclovir, into a phosphorylated nucleoside analog that blocks DNA replication and thereby further growth. The phosphorylated ganciclovir bystander effect has recently been shown to be the consequence of gap junction - mediated transfer of the toxin to adjacent cells.

However, the benefit of the bystander effect in treating animal models of solid tumors has required the administration of viral producing cell lines to yield adequate transduction with the consequence of measurable reductions in tumor mass. This strategy was first reported by Culver et al for the treatment of brain tumors engrafted in nonhuman primates. In this report, a murine cell line that produced a recombinant retrovirus containing a viral thymidine kinase gene was administered to the engrafted tumor mass, the concept being that the tumor cells would be continually exposed to new virus over an extended period of time. The animals were subsequently treated with ganciclovir and demonstrated dramatic reductions in tumor mass.

The producer cell line approach does have important limitations. First, the producer cells are xenografts of immortalized cells that may be quickly eliminated by host immune mechanisms. Second, this method relies on the existence of stable producer cell lines that are available only for recombinant retroviruses, but not for other recombinant viruses. The recombinant adenoviruses, adeno-associated viruses, and herpes viruses are all produced by lytic infections of their corresponding "packaging" cell lines. Thus, these viruses cannot be utilized for this type of cancer therapy. Third, in a trial of recombinant retroviral producer cell treatment of brain cancers, multiple injections were required to produce responses.

The clinical applications of gene therapy would be greatly advanced by the development of new approaches to achieving high levels of gene transduction *in vivo*. A particularly valuable approach would be one that provides for the continuous production of new recombinant vector that is applicable to multiple vector systems and does not require the administration of xenogenic cells.

It is therefore an object of the present invention to provide vectors for gene therapy and methods for use thereof not requiring xenogenic cells.

It is a further object of the present invention to provide
5 improved defective viral vectors that can be co-transduced with elements for intracellular reproduction of the defective viral vectors, which are capable of infecting other cells.

SUMMARY OF THE INVENTION

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Replication-defective viruses and means for intracellular replication thereof are described which are useful for gene therapy. Human cells can be changed into recombinant virus-producing cells by the simultaneous delivery to those cells of two different nucleic
15 acids: the first being a replication-defective viral genome, the second being a nucleic acid that complements the viral sequences deleted from the first nucleic acid so as to result in the production of new infective virus. The first nucleic acid can be delivered by the replication-defective virus itself, or as a nucleic acid that is not part of
20 the virus. In a preferred embodiment, the replication-defective virus includes elements to maintain the two nucleic acids in combination during transduction.

Examples of preferred viral sources are adenoviruses, herpesvirus, retroviruses, and adeno-associated viruses. Nucleic acids
25 useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins to treat a pathophysiologic condition. Commonly, the second nucleic provides sequences that in a
30 trans configuration enable the first nucleic acid to replicate and be packaged into new, replication-defective viral particles can also contain other nucleic acids that are useful for gene therapy. A preferred example of a linking means for the two nucleic acids is to conjugate on the surface of the defective virus containing the first
35 nucleic acid highly charged polyamino acids, such as polylysine, which binds ionically to the second nucleic acid.

The present invention further shows that cationic lipid-facilitated delivery of the replication-enabling plasmid and free E1-

defective adenovirus achieved new E1-defective adenovirus production. Two new plasmids were developed for the delivery of the replication-enabling functions, and conditions identified whereby amounts of E1-defective virus produced were comparable to that
5 achieved with the original replication-enabling plasmid while eliminating all detectable RCA production.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Figures 1A and 1B are schematics of the infection and replication processes of replication-competent virus (Figure 1A) and replication-incompetent, or defective, virus (Figure 1B).

Figures 2A and 2B are maps of the complementary adenoviral nucleic acid sequences. Figure 2A is a map of the
15 AdCMVlacZ viral genome. Hatched bars represent the conserved human type 5 adenovirus sequences with map units indicated below. CMV promoter sequences ("CMV"), bacterial lac Z sequences ("lac Z"), and SV40 polyadenylation signal ("pA") were inserted as a cassette in place of the deleted E1A and E1B sequences. Figure 2B is a map of
20 pE1A. Adenovirus type 5 nucleic acids are shown ("Ad 5") with nucleotide numbers above and map units below the indicated fragment. The Ad 5 nucleic acids were ligated into the multiple cloning site of pUC 13 represented by hatched bars at the indicated restriction endonuclease sites. "E" = Eco RI, "B" = Bst 1107 I, "H" = Hinc
25 II.

Figure 3 is a graphic representation of the observed cytopathic effects produced by lysates or supernatants from cells cotransduced with AdCMVlacZ and plasmid DNA. Ordinate = qualitative amount of cytopathic effect 48 hrs after addition of the
30 supernatants or lysates, abscissa = times of lysate or supernatants after cotransduction. Key indicates the plasmid cotransduced with the adenovirus. The pUC 13 groups were observed up to 96 hrs after supernatant or lysate addition without any cytopathic effect.

Figures 4A and 4B are maps of the retroviral nucleic acids.
35 Figure 4A is a map of pBAG. "LTR" = long terminal repeat, "b-galactosidase" = bacterial lac Z gene, "neo" = neomycin resistance gene, "pbr origin" = plasmid origin of replication. Not to scale. Figure 4B is a schematic of retroviral sequences within the pPAM3 plasmid. Open

blocks at left indicate the 5' LTR sequences, "SD" = splice donor site, "SA" = splice acceptor site, "gag, pol, env" = retroviral coding sequences, hatched bar = SV40 polyadenylation signal. (Reproduced from (Miller 1986 Molecular and Cellular Biology 6(8):2895.).

5 Figure 5 is a graphic representation of the titers of retrovirus in supernatants of cells cotransduced with pBAG and pPAM3. Supernatants were collected 72 hrs post-transduction with the complementary retroviral vectors. Ordinate = titer as number of viral particles per ml using A549 as indicator cells, abscissa = amounts
10 of the 2 plasmids used to make the molecular conjugates. The cell type cotransduced with the two plasmids were A549 (circles) and PC-3 (triangles).

 Figure 6 shows variables modulating new virus production by the cationic lipid-mediated delivery of replication-enabling plasmid DNA and the E1-defective virus, AdCMV-luc. Figure 6a shows
15 the influence of multiplicity of infection (moi) on new virus production. Shown is the mean of 3 experiments S.E.M.. Ordinate: plaque forming units (PFU) detected on 293 cells per ml of lysate; abscissa: moi of AdCMV-luc. Plasmid cotransduced with AdCMV-luc is
20 indicated by the key above. Figure 6b shows the influence of plasmid dose on new virus production. Shown is the mean of 3 experiments S.E.M. Ordinate: plaque forming units (PFU) per ml of lysate in cells cotransduced with pE1 and AdCMV-luc; abscissa: plasmid amount added to each well. pUC 13 (1 g) addition as a negative control
25 produced 3515 PFU/ml lysate (not shown for clarity).

 Figure 7 shows that adenoviruses present in lysate of cells cotransduced with the pE1 replication-enabling plasmid and E1-defective virus as quantified by 293 and HeLa cell plaque assays. Ordinate: plaque forming units (PFU) per ml lysate; abscissa: plasmid
30 cotransduced with the virus. Legend above indicates cells on which plaques were produced. * = no HeLa plaques detectable.

 Figure 8 shows that adenovirus present in lysate of cells cotransduced with AdCMV-luc and CMV-directed E1 encoding plasmids. Ordinate: plaque forming units (PFU) per ml lysate S.E.M.,
35 abscissa: plasmid(s) cotransduced with virus. CMVE1A/E1B indicates transduction of both pCMV-E1A and pCMV-E1B.

 Figure 9 shows that new replication-enabling plasmids. Above: pUC-E1A, a plasmid containing adenovirus serotype 5 E1A

encoding region. Open bar represents the adenovirus sequences with nucleotide numbers shown above along with relevant regulatory signals. Dashed lines represent pUC backbone with restriction enzyme sites of insertion indicated by letters. E = Eco RI, X = Xho I. Below:
 5 pUC-E1B, a plasmid containing adenovirus serotype 5 E1B-encoding region. Hatched bar represents the adenovirus sequences with nucleotide numbers shown above along with the relevant regulatory signals. Dashed lines represent pUC backbone with restriction enzyme sites of insertion indicated by letters. B = Bam HI.

10 Figure 10 shows that virus produced by the new replication-enabling vectors in varying ratios. Shown is the mean of 3 expts. Ordinate: plaque forming units (PFU) per ml lysate; abscissa: plasmid(s) cotransduced with the E1-defective virus with pUC-E1A:pUC-E1B ratios shown.

15 Figure 11 shows that detection of amplified replication-competent adenovirus (RCA) in lysates of cells cotransduced with E1-defective adenovirus and plasmid. Shown is the result of a typical experiment (n=3). The plaque forming units (PFU) detected in 293 cells (abscissa, bottom) were determined with the lysates from the
 20 cotransduced cells. The PFU detected in the A549 cells (abscissa, top) represent the amplified RCA detected in lysates of cells 3 days after exposure to the lysates from the cotransduced cells. Ordinate: plasmid(s) cotransduced with the E1-defective virus (AdCMV-HSVTK). "AB, ratio"=pUC-E1A+pUC-E1A in the ratio shown. Amount of plasmid
 25 cotransduced indicated by brackets at left. * = no plaques detected in the amplified RCA assay for those groups.

Figure 12 shows the amplification of viral TK-mediated killing of cervical adenocarcinoma (HeLa) cells in vitro. Ordinate: numbers of cells per well, abscissa: percentage of cotransduced cells
 30 (AdCMVHSV-tk virus + plasmid) mixed with naive HeLa cells. Solid bars are groups that received the control plasmid with the virus, hatched bars are groups that received the virus and the replication enabling plasmid.

Figure 13 shows the amplification of viral -TK mediated
 35 killing of cervical adenocarcinoma (HeLa) cells in vivo. Ordinate: mass of tumors, abscissa: plasmid cotransduced with the virus numbers in parentheses indicated the percentage of cotransduced cells mixed with the naive cells. n=3 per group, values shown are \pm S.E.M.

Figure 14 shows that 13s E1A RNA transcript replication enable E1-defective adenovirus. Shown is adenovirus quantified in lysates of HeLa cells cotransduced with AdCMVHSV-tk and the nucleic acids indicated on the abscissa. Lane 1: 0.1 μ g 13s RNA + 0.9 μ g pUC-E1B; Lane 2: 0.5 μ g 13s RNA + 0.5 μ g pUC-E1B; Lane 3: 0.9 μ g 13s RNA + 0.1 μ g pUC-E1B; Lane 4: 4.5 μ g 13s RNA + 0.5 μ g pUC-E1B; Lane 5: 0.5 μ g pSVN20 + 0.5 μ g pUC-E1B; Lane 6: 1.0 μ g 13s RNA; and Lane 7: 1.0 μ g pUC-13. Adenoviral sequences from pSVN20 were excised by restriction endonucleases Eco RI and Pst I, the fragment isolated and overhanging ends blunted with T4 DNA polymerase using standard techniques. The pSP64T was linearized by Bgl II digestion and the overhanging ends blunted by Klenow fragment. The plasmid and adenoviral sequences were ligated and positive transformants identified by colony hybridization using standard techniques. Transcripts encoding the E1A 13s protein product were generated by linearizing pSP64T-13s with Bam HI and the linearized plasmid used to generate capped RNA transcripts by activation of the SP6 promoter using the reagents and instructions of a kit (Message Machine™, Abion, Inc., Austin, TX). The transcripts were analyzed qualitatively in the denaturing agarose gels using previously described conditions. RNAs and/or plasmids were complexed with DOTA/DOPE and cotransduced as described above.

Figure 15 shows the luciferase transfer capacity in the supernatants of HeLa cells cotransduced with DOTAP/DNA complexes containing AdCMVluc DNA and pE1A plasmid DNA. Shown are the results 6 days after exposure to the DOTAP/DNA.

Figure 16 shows the luciferase activity present in engrafted PC-3 tumor 10 days after they were injected with either AdCMVluc and pE1A, or for the controls, AdCMVluc and pUC13.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "replication" is the process of producing new viral particles that are capable of infecting other cells. Infection is the process of a viral particle binding to a specific cell surface receptor resulting in the release of the viral genome into the nucleus where the viral genome directs the production of new proteins.

As shown by Figure 1A, a replication-competent virus infects a cell when the viral particle binds to a receptor on the surface of the cell and is transported into the cytoplasm and then into the nucleus. Once in the nucleus, the complete viral genome is transcribed, new viral proteins are synthesized, and new viral particles are made and released. Figure 1B shows a replication incompetent virus with the viral particle containing an incomplete genome also binds to a cell surface receptor and is transported through the cytoplasm into the nucleus. However, since the viral genome is incapable of either being transcribed, or directing synthesis of all viral proteins required for packaging, infection with a replication-defective virus does not lead to new viral particle production.

The production of syngeneic, replication-defective, recombinant virus-producing cells requires several steps. The starting point is the requirement for a replication-defective virus that can produce new replication-defective virus in the presence of additional genes provided in a trans configuration. Although the specific designs of these different viruses vary, generally the recombinant viral genome has some of the native viral genes required for replication deleted, which may be replaced by new genes. The replication-defective virus includes the 5' and 3' LTRs and packaging signal sequences. This replication-defective virus is generally referred to herein as the "first nucleic acid sequence".

The second step is the design of complementing genomes that are co-delivered to the target cells in order to produce new recombinant virus. These complementing genomes must be present in the cells in which new recombinant virus is to be produced but should preferably not recombine with the defective virus to produce replicating virus. The complementing genome is generally referred to herein as the "second nucleic acid sequence". As part of either the first and/or second steps, the biologically active genes of interest delivered and expressed in the target cells are incorporated into either, or both, the first and second nucleic acid sequences, most preferably the first nucleic acid sequence. An optional third step is to provide means for co-delivering the first and second nucleic acid sequences.

The final step is to co-deliver the first and second nucleic acid sequences. This can be accomplished by multiple, widely available gene transduction methods. One then must establish that co-delivery of the first and second nucleic acids results in the production of new recombinant virus which is infectious and capable of expressing the incorporated genes of interest in the target cells. The presence of new recombinant virus can be assayed in the supernatant of the cells transduced several days later, or within lysates of the transduced cells. The identity of the new virus can be established by the presence of marker genes, or marker gene products, within the recombinant virus genome.

Replication-Defective Virus ("RDV")

Many replication-defective viruses, particularly derived from retroviruses, adenoviruses, adeno-associated viruses, and herpes viruses, have been described and are available from, e.g., the American Type Culture Collection, Rockville, Maryland. These viruses are characterized as missing all or a portion of one or more genes essential for replication of the virus. In an appropriate cell line, however, they are capable of forming infectious virus particles. The viruses useful in the methods described herein are those capable of infecting and replicating in mammalian cells, although they may infect certain cell types preferentially.

Retroviruses are generally defined as a family of eukaryotic viruses that replicate through a DNA intermediate, as described by Panganiban 1985 Cell 42:5. Herpes virus are classified based on a characteristic virion architecture that includes an icosadeltahedron forming the capsid with two-fold symmetry surrounding the tegument that surrounds the DNA genome, (Roizman in Human Herpesviruses 1993, Raven Press, pp. 1-9). Adenovirus are nonenveloped viruses containing a double stranded DNA genome with characteristic antigenic properties and DNA homology, as described by Wadell 1984 Current Topics in Microbiology 110:191. Parvoviridae are a family of DNA, non-enveloped animal viruses containing a single stranded DNA genome encapsulated within an icosahedral protein coat composed of three proteins with overlapping amino acid sequences. The family includes three genera that includes (i) parvoviruses (ii) adeno-associated viruses (AAV) that usually require coinfection with

adenovirus (iii) densoviruses which multiply in insects, as described by Berns 1990 Microbiological Reviews 54:316.

Recombinant retroviruses are generally made by manipulating the proviral form of the virus, i.e., the double stranded
5 DNA copy of the viral RNA genome. The starting virus is commonly the Maloney murine leukemia virus that is available from ATCC. Examples include VR-861, VR-860, VR-590, and VR-589. The proviral DNA is placed within a plasmid to permit amplification of the DNA in bacteria. Once this has been accomplished, the gag, pol and
10 env genes are excised by appropriate restriction endonucleases to leave the 5' and 3' long terminal repeats (LTRs), and the packaging signal sequences that are immediately 3' to the 5' LTR. Multiple, detailed descriptions of these constructions, as well as permutations of this general scheme, are described in, for example, by Cepko 1984 Cell
15 37:1053; Hwang 1984 J. Virol. 50:417; Yu 1986 Proc. Natl. Acad. Sci. USA 83:3194; Armentano 1987 J. Virol. 61:1647.

Recombinant adenoviruses are generally made by manipulating the adenoviral DNA within plasmids to permit amplification of the DNA in bacteria. To date, the human serotypes 2
20 and 5 have been used for gene therapy purposes, largely because their biology and genomes have been most extensively characterized. The wild type adenovirus type 2 and 5 are readily available from the ATCC: Type 2, VR-846 and VR-1079; Type 5, VR-5 and VR-1082. Most commonly, the E1A region is deleted using convenient restriction
25 endonuclease sites within the E1A region. Often, a portion of E3 is also deleted by restriction endonuclease addition so as to permit the insertion of a larger piece of foreign DNA while still satisfying the size constraints required for packaging into new viral particles. The details of andoviral vector constructions are widely described in, for
30 example, by Berkner 1984 Nuc. Acids Res. 11:6003; Ghosh-Choudhury 1987 Biochem. Biophys. Res. Commun. 147:964.

Traditionally, these replication-defective viruses have been replicated through the use of packaging cell lines. These cell lines contain, at a minimum, the nucleic acid sequence obtained from
35 replicating virus that will complement in trans the replication-defective viral genome, resulting in new replication-defective virus. A packaging cell line is made by stably introducing the missing viral genes that are required for replication. Recombinant virus is made by

introducing the recombinant viral genome into the packaging cell line, the viral genome (minus the genes present in trans that complement) is replicated and packaged into viral particles that can infect any cell type with the required viral receptor. However, since the virus is still
5 replication-defective, the viral particles are incapable of directing the production of new virus.

It has not previously been established whether other elements in the specific packaging cell lines might contribute to the production of virus in unpredicted or unexpected ways. For example,
10 the 293 cell line used for recombinant adenovirus production were made by the introduction of randomly sheared adenoviral DNA that has been only partially characterized (Graham 1977 Journal of General Virology 36:59; Aiello 1979 Virology 94:460). Although it has been shown that these cells contain E1A genes, and are capable of
15 "packaging" E1A-deleted recombinant adenoviruses, it was never established whether other sequences also present played a role, whether the copy number of the sheared viral nucleic acid sequences present in each cell was critical to the utility of this specific cell line, or whether the specific parent cell type contributed to the virus
20 production. Similarly, although the retroviral nucleic acids used for establishing the PA317 packaging cell line were precisely identified (Miller 1986), it was unclear whether these sequences had to be stably integrated within a cell line before the addition of the recombinant viral genome, i.e., it was never established that co-
25 delivery of the two complementing genomes would result in the production of recombinant viral particles.

Some recombinant viral vectors are used to produce recombinant virus particles by coinfection with a helper virus, but the specific helper virus genes that are required to replication-enable the
30 viral vector have not been defined. Specific examples of such vectors include the adeno-associated virus vectors described by Nahreini 1993 Gene 124:257; and Samulski 1989 J. Virol. 63:3822, and herpes vectors, described by Geller 1988 Science 241:1667; and Breakfield 1991 New Biol. 3:203.

35 A replication-defective adenoviral genome was delivered by viable virus in the specific examples described below. Alternatively, the same nucleic acids could be delivered in a plasmid form, since the methods for propagation of part (Berkner 1983 Nucleic

Acids Research 11:6003), or all of the adenoviral genome (Graham 1984 EMBO Journal 3:2917) within plasmid DNA have been previously described.

Genes required for replication of replication-defective virus

5 In general, it is straightforward to supply the genes missing in the replication-defective virus (RDV) in order to provide a second nucleic acid sequence that operates in trans with the RDV to replicate the RDV. Although the second nucleic acid sequence can include nucleic acid sequence also present in the RDV, it is preferred
10 that there not be overlapping sequence since this can cause an undetermined amount of recombination, leading to wild type virus capable of replicating. This is not in itself necessarily a problem, however. As referred to herein, the second nucleic acid sequence includes at a minimum the genes required for replication of the
15 replication-defective virus and means for amplification thereof. It may optionally include genes of interest, such as marker genes, suicide genes, and therapeutic genes. Although referred to as a "sequence", the parts of the second nucleic acid sequence can be present in one or more molecules, usually plasmids, preferably a
20 single plasmid.

In general, the genes required for replication ("GRR") can be obtained by amplifying some or all of the viral nucleic acids present in a packaging cell line useful for replicating the RDV. The genes required for replication can also be obtained by excision from
25 virus that is capable of replication. For example, in the case of recombinant adenovirus, the viral nucleic acids that contain the E1A region are excised, subcloned and amplified by routine techniques. These nucleic acids complement the deleted E1A sequences in common recombinant adenoviruses heretofore only provided by
30 packaging cell lines, such as the 293 cell line, for the purpose of replicating the viruses. In the case of recombinant retrovirus, the nucleic acids that are used to make the packaging cell line are obtained in plasmid form and amplified with the intent of co-delivery with the recombinant retroviral vector plasmid.

35 The second nucleic acid sequence must be provided in a form that can be amplified, although it is preferably not in a form that infects cells other than the targeted cells which are transduced with the first nucleic acid sequences. By using a second nucleic acid

sequence which is limited to defined cells, replication and infection of cells can be controlled. This is particularly important in the case where the genes of interest incorporated with the first and/or second nucleic acid sequences result in the death of the host cells. The most preferred form therefore is a plasmid. However, the second nucleic acid could also be provided within a recombinant virus, preferably one utilizing different receptors than a virus containing the first nucleic acid. In all cases, the second nucleic acid must include genes with the necessary transcription activating and terminating elements necessary to transcribe mRNA that can be translated into proteins capable of enabling replication.

In the preferred embodiment where the means for amplification of the genes required for replication is a plasmid, the plasmid promoter should allow replication in the cells to be transduced with the second nucleic acid sequence. The promoter may be cell type or tissue type specific as another means for controlling which cells are targeted.

In the following examples, the E1A genes required for replication of adenovirus are provided in the pE1A plasmid. The construction of this plasmid is described in detail in the example using pEco RIA plasmid described by Berkner 1984 Nuc. Acids Res. However, the E1A fragment or other complementing fragments that might be needed for replication-enabling other adenoviral vectors, could easily be derived from adenoviral DNA that is harvested from adenovirus-infected 293 cells, or other adenoviral-permissive host cells.

The genes required for replication of retrovirus are provided in the pPAM3 plasmid described in the following examples. The construction of this latter plasmid is described in detail by Miller 1986 Mol. Cell. Biol. 6:2895. This description could be applied by one of ordinary skill in the art to construct an equivalent plasmid from the murine leukemia virus proviral DNA.

Genes to be incorporated into First or Second Nucleic Acids

Nucleic acids useful for gene therapy include those that code for proteins used to identify cells infected with the recombinant virus, those that encode for proteins that function to kill cells containing the viral genome, or that encode for therapeutic proteins that will serve to treat a pathophysiologic condition within the body.

The sequences that encode for many of these proteins are known and published in the literature. Representative marker genes include those described in detail in the following examples, including an enzyme such as galactosidase and proteins conferring antibiotic resistance or susceptibility. Other examples include proteins that augment or suppress abnormal proteins, as well as those that are toxic or deleterious to abnormal cells within the body. One example of the latter is the herpes simplex virus thymidine kinase gene. The addition of ganciclovir to cells expressing this gene results in death of the cell. Still others are those which are defective or missing in the patient to be treated, for example, the cystic fibrosis transmembrane regulator gene ("CFTR") can be added to cells containing mutant CFTR with subsequent correction of the ion transport defect caused by the mutant CFTR gene. Examples of other genes currently being investigated for use in gene therapy include adenosine deaminase, insulin, coagulation factors such as factor VIII, and glycogen degrading enzymes.

Although the sequences incorporated into the first and/or second nucleic acids will typically be nucleic acids encoding proteins, the sequences themselves may also be biologically active. Many examples of such materials are known, for example, antisense and ribozymes. Unless specifically stated otherwise, the genes encode therapeutic molecules including biologically active nucleic acids, nucleic acids encoding biologically active proteins, and nucleic acids encoding proteins responsible for producing the biologically active molecules of interest, whether protein or other type of molecule.

For example, for human gene therapy use, one of the retroviral nucleic acid constructs could be modified to contain a "suicide gene" so that the virus producing cells could be eliminated as desired. As one specific example, the neomycin resistance coding sequences could be excised from pBAG, described in detail in the following examples, with appropriate restriction endonucleases and replaced with herpes simplex virus thymidine kinase coding sequences. It is well established that cells expressing the viral thymidine kinase gene product can be eliminated by treatment with the antiviral agent, ganciclovir (Moolten 1986 Cancer Research 46:5276). In this manner, the virus producing cells would be eliminated by systemic administration of FDA-approved ganciclovir.

Means for Co-Delivery of First and Second Nucleic Acid Sequences

As described herein, the method for gene therapy can be *in vivo*, i.e., administered directly to a patient for expression of a gene as defined above or for targeted killing of cells, or *in vitro*, for administration directly to cells outside of the body, for expression of exogenous genes. In a preferred example of the latter, cells are obtained from a patient, the first and second nucleic acid sequences administered to the cells, and the cells returned to the patient. An example of this method is the treatment of stem cells or progenitor cells obtained from a patient following cytokine administration to enhance proliferation and mobilization of the stem cells and progenitor cells in the peripheral blood. The genetically engineered cells are then returned to the patient. Alternatively, the cells can be maintained in culture for production of the molecules encoded by the exogenous genes carried by either the first and/or second nucleic acid sequences.

In general, preferred means for co-delivery include the use of (1) a first nucleic acid sequence including infective replication-defective virus in combination with the second nucleic acid sequence, (2) the first nucleic acid sequence ionically or covalently coupled to the second nucleic acid sequence, alone or in further combination with an enhancer of transduction, (3) intact infective virus in combination with the first nucleic acid sequences coupled with the second nucleic acid, where the intact virus is rendered non-viable after infection by ultraviolet irradiation in the presence of 8-methoxypsoralen or other free radical initiators such as methylene blue, and (4) delivery of both the first and second nucleic acid sequences separately, by viruses utilizing different receptors.

In the preferred embodiment, the first and second nucleic acid sequences are introduced simultaneously into the cells where virus is to be replicated. The first nucleic acid within a virus is delivered to the cells by the viral infection medium, and the second nucleic acid is added separately by a non-viral means, for example, naked DNA administration or coupled with an enhancer of transduction. The second nucleic acid can also be coupled to the virus containing the first nucleic acid by means of an agent on the viral surface having an affinity for nucleic acids, such as polylysine, followed by the addition of free agent to further condense the second

nucleic acid, after which the entire complex is administered to the cells. Alternatively, the second nucleic acid can be coupled to an inactivated virus by means of an agent on the viral surface having an affinity for nucleic acids by first inactivating the virus followed by all
5 the other steps used to make a complex as described above. The first and second nucleic acids can also be coadministered by viruses utilizing different receptor types.

Infection with the defective retrovirus:

For example, retroviral-mediated delivery of the E1A
10 sequences can be accomplished using the pLN retroviral vectors developed by Miller and colleagues (Miller 1989 BioTechniques 7:980), provided A.B. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA. The desired E1A sequences are excised with appropriate restriction endonucleases from a parent plasmid construct, blunted
15 and ligated into a similarly blunted cloning site of pLNSX, described by Miller, 1989 BioTechniques 7:980. Since pLNSX contains an SV40 early promoter upstream of the cloning site, this can be excised by a *Bam* HI/*Hind* III digest at the time of linearizing the vector, after which the sites are be blunted. The method described by Butterworth
20 and Miller (Miller 1986) can be used in which the plasmid retroviral vector containing the E1A sequences is transfected into the Psi2 ecotropic packaging line described by Cepko 1984 Cell 37:1053, without selection. The transient supernatant containing some ecotropic virus is collected 48 hours later and used to infect the
25 PA317 ecotropic packaging cell line by Miller 1986 Mol. Cell Biol. 6:2895. Individual producer clones are selected by culturing the cells in G418 media, amplifying and testing for titer by methods described by Rousculp 1992 Human Gene Therapy 5:471.

Co-delivery of first and second nucleic acid sequences:

30 Examples of materials facilitating co-delivery of the first and second nucleic acid sequences include charged molecules such as polylysine, that ionically couple the nucleic acid sequences, as well as biotin conjugation with strepavidin and other cross-linking agents that can be used with standard technology to covalently couple the
35 first and second nucleic acid sequences. Coupling by means of an agent ionically binding nucleic acid, such as polylysine is also contemplated.

DNA can be coupled using a covalent crosslinking agent, for example, polyglutaraldehyde (Digene Diagnostics), or more typically, by biotinylation and linkage via an avidin (streptavidin) bridge to the viral surface. Biotinylation is well known to those skilled in the art, for example, as described by Avignolo, et al., 1990 Biochem. Biophys. Res. Commun. 170:243-250.

Example 1 demonstrates a preferred embodiment for co-delivery of pE1A (a second nucleic acid sequence consisting of a plasmid and the genes required for replication for a replication-defective adenovirus) and AdCMVlacZ (a replication-defective adenovirus including the CMV promoter and lacZ bacterial gene encoding galactosidase as a marker gene) accomplished by ionically linking the pE1A to the virus exterior. Figure 2A is a map of the AdCMVlacZ viral genome. Hatched bars represent the conserved human type 5 adenovirus sequences with map units indicated below. CMV promoter sequences ("CMV"), bacterial lac Z sequences ("lac Z"), and SV40 polyadenylation signal ("pA") were inserted as a cassette in place of the deleted E1A and E1B sequences. Figure 2B is a map of pE1A. Adenovirus type 5 nucleic acids are shown ("Ad 5") with nucleotide numbers above and map units below the indicated fragment. The Ad 5 nucleic acids were ligated into the multiple cloning site of pUC 13 represented by hatched bars at the indicated restriction endonuclease sites.

Molecular conjugates can also be used to deliver the E1A nucleic acid sequences. These consist of a means for ionically linking the first and second nucleic acid sequence via a covalently coupled conjugate. One example is the polylysine-conjugated transferrin (TfpL) commercially available from SIGMA Chemical Co., St. Louis, MO (Cat. # T 0288). The optimal proportions of nucleic acid to TfpL are established in preliminary experiments with a reporter plasmid. The E1A sequences are mixed with the TfpL and added to the target cells. Infection with a virus then rendered non-viable

A virus capable of normal infection can be rendered non-viable by exposure to a dye such as 8-methoxypsoralen which kills virus in the presence of ultraviolet radiation, as described in detail by Cotten, et al., 1992 Proc. Natl. Acad. Sci. (USA) 89:6094, after which the first and/or second nucleic acids can be coupled to the inactivated surface as described above. Other useful materials include methylene

blue and other free radical initiators and dyes that are known to selectively damage viral DNA.

Transduction Enhancers

5 The first and second nucleic acid sequences may be transduced separately or together into the cells where viral replication is to occur. There are many methods for transduction of cells with viral material that are known to those skilled in the art, for example, Gene transfer and expression: a laboratory manual Kriegler M. 242 pp. (W.H. Freeman, NY 1991); and Current Protocols in
10 Molecular Biology, 1987-1994, Ausubel F.M., et al., section entitled "Introduction of DNA into Mammalian Cells" pp. 9.0.1-9.17.2 (John Wiley & Sons).

In a preferred example of a transduction enhancer, cationic lipids that are commonly modified phospholipids to obtain a
15 positive charge are used for transduction of the first and second. A number of lipid compounds shown to have efficacy for nucleic acid transduction, for example, the cationic lipid N-[1-(2,3-Dioleoyloxy) propyl]-N,N,N-trimethyl-ammoniummethylsulfate (DOTAP) can be used. The stock solution commercially obtained from Boehringer
20 Mannheim is diluted three-fold with HEPES buffered saline and mixed with DNA at 50 ng/ml. The typical ratio of lipid to DNA (weight:weight) is 6:1, but other ratios may provide better results as determined by empiric testing. After allowing the DNA and lipid to associate for 10 minutes, the lipid-DNA complexes are administered to
25 the target cells.

In each of these alternative delivery methods, the precise conditions for the cotransduction can be easily established by those skilled in the art using the teachings provided here. In certain cell types, optimal results are obtained by separating the infection with
30 the virus from the transduction of the complementing GRR sequences. In addition, some cell types or physiologic situations may produce optimal results with a specific means of GRR nucleic acid delivery. Furthermore, it is possible that multiple transductions of the GRR and/or the RDV may result in additional virus production.

35 Co-transduction of replication-defective adenovirus

This overall strategy was employed in the following examples demonstrating the successful development of compositions

and methods for making recombinant adenovirus from cotransduced cells that prior to transduction did not make any virus or contain any virus nucleic acids. The recombinant adenovirus employed was AdCMVlacZ (Yang 1993 Proc. Natl. Acad. Sci. (USA) 90:4601), an E1A-
5 deleted virus (map units 1.3 - 9.4 deleted) that included a CMV promoter-driven, bacterial lac Z gene. A viral stock of AdCMVlacZ was amplified in 293 cells and modified to contain polylysine molecules on the virus exterior using methods described in detail in Example I. A plasmid, designated pE1A, was designed to contain the E1A nucleic
10 acids deleted from AdCMVlacZ. The pE1A plasmid contained nucleotides 1- 5768 of the human adenovirus type 5 genome that included E1A and E1B. Although the pE1A plasmid was derived from another adenoviral plasmid, the required nucleic acids could be easily derived from adenoviral infected cells, or directly from adenoviral
15 DNA that does not contain any deletions in the E1A region.

The possibility of recombination can be eliminated, or greatly reduced, by making a complementary plasmid that contains the bare minimum of sequences necessary to complement those missing from AdCMVlacZ, or related E1A-deleted viruses. Although
20 the E1A region encodes proteins of 243 and 289 amino acids (Flint 1989 Ann. Rev. Genetics 23:141), previous work has shown that the 289 amino acid protein alone is sufficient for trans activating the other viral genes and establishing lytic infection (Winberg 1984 EMBO Journal 3:1907; Moran 1986 J. Virology 57:765). Very recently, exon
25 2 of the E1A region was shown to be sufficient for transactivation of the other viral genes, although the degree of activation was variable in different cell lines (Mymryk 1993 J. Virology 67:6922). Therefore, one can utilize smaller portions of the E1A region than employed in the following examples. The variation of trans activation by exon 2 of
30 E1A in different cell types can also be used to target viral replication to cell types in which exon 2 of E1A alone is a sufficient trans activator.

Although one recombinant adenovirus is used in the following examples, it is evident that the approach can be used for
35 trans complementation of other adenoviral genes, and other adenoviral serotypes. For example, fibre-deleted adenoviral mutants and methods for propagating those mutants have been described by Falgout 1987 J. Virology 61:3759; Falgout 1988 J. Virology 62:622. In

the case of the fibre-deleted mutant, the strategy would employ the co-delivery of the recombinant virus with the deleted fibre gene and a separate nucleic acid encoding the deleted fibre sequences. Since trans complementation of replication-defective adenoviruses using
5 cell lines that contain complementing nucleic acids has also been described for E4 and E2A deletion mutants (Weinberg 1983 Proc. Natl. Acad. Sci. (USA) 80:5383; Klessig 1984 Mol. Cell. Biol. 4:1354), these are additional replication-defective adenoviruses that could be complemented in trans using the methods described here.
10 Furthermore, this type of approach could be used to mix genes of different adenoviral serotypes to produce new tropisms or other biological effects.

Co-transduction of replication-defective retrovirus

Recombinant retrovirus was also produced by cells
15 cotransduced with recombinant viral and complementing viral nucleic acid sequences. The recombinant retroviral vector used in the example shown here, pBAG, contains a bacterial lac Z gene and a neomycin resistance gene in place of deleted viral genes gag, pol and env (Price 1987 Proc. Natl. Acad. Sci. (USA) 84:156). A plasmid vector
20 containing the deleted retroviral genes, pPAM3, was chosen as the complementing nucleic acid because this plasmid had been used to create the widely used packaging cell line, PA317 (Miller 1986). Both the vector and complementing plasmids were co-delivered by ionic linkage to polylysine conjugated to the exterior of the DL1014
25 adenovirus (Bridge 1989 J. Virology 63:631) using the methods detailed in Example 2.

Co-transduction of retrovirus and adenovirus genes

The examples described below are specific to either replication-defective adenovirus or to replication-defective retrovirus.
30 An advantage of retrovirus is that it only infects replicating cells, as reported by Miller 1990 Molecular and Cellular Biology 10(8):4239. This feature has been used to target replicating cells in a therapeutic context, as described by Culver 1992 Science 256:1550. This is particularly useful in cancer treatment since the tumor cells typically
35 replicate more rapidly than the normal host cells. In the brain, the tumor cells are typically the only replicating cells. Accordingly, one can use a combination of retrovirus and adenovirus to replicate replication-defective virus selectively in replicating cells. The

retrovirus still infect only replicating cells. It is used as a carrier for the GRR for adenovirus (E1A). The cells are also infected with replication-defective adenovirus. Since the purpose of infecting the cells is to kill the cells, and infection and replication of adenovirus in cells results in cell lysis and death, no additional non-viral genes are required to kill the targeted cells.

In addition to the methods described above, recombinant adenoviruses containing one or both of the complementary retroviral nucleic acid sequences can also be constructed and used. In this case, the replication-defective virus is a retrovirus and the replication-defective adenovirus is a carrier for the GRR for the retrovirus. The methods described by Graham and Prevec (1991) Methods in Molecular Biology. Clifton, The Humana Press Inc. 109, are used to create replication-defective adenoviruses containing the retroviral nucleic acids at the site of the E1A deletion. In brief, the retroviral nucleic acid sequences are ligated into a blunted cloning site of pXCJL-2 plasmid that contains the E1A deletion portion of the virus. Standard techniques are used for the plasmid construction and identification. The recombinant virus is made by co-transfecting the 293 cell line (which provides E1A viral proteins in trans) with the pJM17 vector that is designed to homologously recombine with the pXCJL plasmid derivatives to produce a full length, packagable viral transcript lacking E1A, as described by Graham 1991.

The following non-limiting examples demonstrate actual reduction to practice of the methods and compositions described herein, as well as preferred embodiments.

EXAMPLE 1

Creation of Syngeneic Recombinant Virus-Producing Cells

A plasmid was constructed to contain the region of adenoviral genome deleted in a replication-defective adenovirus. The replication-defective, human adenovirus, AdCMVlacZ, was unable to replicate in most cells because the lac Z gene had been inserted within the deleted E1A region of the viral genome, as described by Yang 1993. Therefore, the E1A adenoviral region was isolated and inserted into a plasmid vector.

The E1A region was isolated from the plasmid pEcoRIA containing nucleotides 1-27331 of human type 5 adenovirus (Berkner 1983 Nucleic Acids Research 11:6003). Although the pEcoRIA plasmid contained the desired E1A region, it was found in the course of experimentation to undergo frequent recombination events in the course of routine plasmid amplification that reduced its usefulness for the desired application. Adenoviral nucleotide sequences 1-5768 were excised from pEcoRIA by restriction endonucleases *Eco* RI and *Bst* 1107 I. The fragment was isolated from the parent nucleic acid by agarose gel electrophoresis and extracted from the gel using the GeneClean™ II Kit (Bio 101, La Jolla, CA). The blunted E1A-containing fragment was ligated to the *Eco* RI/*Hinc* II site of pUC 13 within the multiple cloning site of that vector. Identity of the final plasmid, designated pE1A, was confirmed by restriction endonuclease analysis that compared observed fragment sizes to those predicted by a computer generated restriction endonuclease map of that fragment.

Adenovirus to be Co-Delivered with Replication-Enabling Plasmid

The AdCMVlacZ virus was amplified by standard techniques by passage through 293 cells. The 293 cells were propagated by routine tissue culture techniques to form an almost confluent monolayer in 10-20, 175 cm² flasks. The cells were inoculated with a stock of the AdCMVlacZ at a multiplicity of infection of approximately 100 to 1000:1 for 2 hrs in a minimal volume of 2% fetal calf serum-containing media. After this period, the tissue culture flasks were supplemented with 10% fetal calf serum-containing media ("regular media") at twice the inoculating volume. Three days later, the infected cells were scraped off the plate and isolated by centrifugation (8000 RPM at 4°C for 30 min in a Beckman JA-17™ rotor). The cell pellet was resuspended in between 5 and 10 ml of regular media, after which the cells were lysed by four consecutive freeze-thaw cycles. The lysate was clarified by centrifugation (8000 RPM at 4°C for 30 min in Beckman JA-17™ rotor) and layered on top of a cesium chloride gradient in Beckman SW-28™ centrifuge tubes. The gradient consisted of 20 ml 1.33 gm/ml cesium chloride in 5 mM HEPES on top of a 10 ml 1.45 gm/ml cesium chloride in 5 mM HEPES cushion. Typically, between 6 and 7 ml of lysate was applied to each tube. The viral particles were concentrated within the

gradient by spinning the tubes for 90 min at 20C at 18,000 RPM. The viral band was extracted by a syringe needle introduced into the side of the tube. The aspirated virus was diluted with an equal volume of 5 mM HEPES, pH 7.8 and applied to the top of a cesium chloride gradient within Beckman SW 41™ tubes. The gradient in these tubes consisted of 3.5 ml 1.33 gm/ml cesium chloride in 5 mM HEPES on top of a 3.5 ml 1.45 gm/ml cesium chloride in 5 mM HEPES cushion. Typically, each tube received between 4 and 4.5 ml of the virus isolated from the first gradient spin. The viral particles were concentrated within the gradient by spinning the tubes for 18 hrs at 20C at 26,000 RPM. After this spin, there was usually a prominent lower band and a fainter upper band. The lower band was aspirated with a syringe needle as described after the first gradient spin.

As one approach to co-delivering the adenovirus and the pE1A plasmid, the adenovirus was prepared to contain a moiety capable of binding plasmid DNA to its exterior. To this end, polylysine was attached to the exterior of the adenovirus isolated as described below. The aspirated virus was adjusted to a volume of 2.5 ml by the addition of 1.33 gm/ml cesium chloride in 5 Mm HEPES. A PD-10™ column (Pharmacia, Piscataway, NJ) was equilibrated with HBS (20 Mm HEPES Ph 7.8, 150 Mm NaCl). The viral solution was loaded onto the PD-10 column by gravity, then eluted with 2 ml of HBS. The column eluate was adjusted to a volume of 3.6 ml by adding HBS. Polylysine solution was made by dissolving 100 mg of polylysine (SIGMA #P-2636) in 10 ml HBS, adjusting Ph to 7.8 with NaOH, followed by adjusting total volume to 16.8 ml by the addition of HBS. 0.4 ml of the final polylysine solution was added to the 3.6 ml of viral eluate. EDC linker solution was made by dissolving 1 gm EDC (Pierce, #22980G) in a total volume of 4 ml distilled water. 40 ml of the final EDC solution was added to the polylysine/virus mixture, rapidly but gently mixed with a pipet, then the mixture incubated on ice for 4 hrs. Following this incubation, 8 ml of cesium chloride in 5 mM HEPES was added, mixed by pipet, and the virus concentrated by centrifugation in an SW 41™ rotor at 25,000 RPM for 18 hrs at 20C. The viral band is again aspirated by a syringe needle as previously described and diluted with an equal volume of viral preservation media (50 % glycerol, 10 mM Tris pH 8.0, 100 mM NaCl, 1 mg bovine serum albumin/ml). The number of viral particles was estimated by

spectroscopy at 260 mμ in which 1 O.D. = 1×10^{12} viral particles. The virus was stored in aliquots at -70°C until further use.

Testing of the Polylysine-Conjugated Adenovirus

It was necessary to test the conjugated adenovirus to
5 establish the optimal number of viral particles and amount of plasmid
to effect maximal expression of coding sequences within the plasmid.
To do this, a range of viral particle numbers: 0.75×10^9 , 1×10^{10} , and
 2.5×10^{10} were diluted to a total volume of 250 ml by the addition of
viral preservation medium. Six micrograms of the constitutively
10 expressed reporter plasmid, pGL2 control (Promega, Madison, WI) was
added to a total volume of 250 ml HBS, and this mixture added to the
viral particles. The DNA was allowed to complex with the polylysine
conjugated to the adenovirus for 30 min at room temperature, after
which 4 mg of free polylysine (stock solution = 1 mg/ml in distilled
15 water) was added to 246 ml of HBS, and the mixture added to the
DNA/adenovirus mix. The free polylysine was permitted to further
complex with the DNA/adenovirus complex so as to further condense
the DNA for an additional 30 min at room temperature. Groups
received either 9 or 12 mg of plasmid DNA and either 6 or 8 mg of
20 free polylysine in order to examine whether higher amounts of
plasmid per viral particle resulted in better expression of the reporter
gene within the plasmid. The final complexes of adenovirus and DNA
were applied to cells that were subsequently tested for reporter gene
expression. The cell lines HeLa, A549, or others were typically plated
25 at densities of $1.5\text{--}2.0 \times 10^5/35$ mm culture dish the night before the
complexes were added. Each plate received 1/10 of the total complex
(i.e., 75 ml) in 1 ml of 2% fetal calf serum. Following a 2 hr incubation
under usual culture conditions of temperature, humidity and CO₂, the
plates were washed 3 times with phosphate buffered saline and
30 replaced with regular media. Two days later, cell lysates were
harvested and analyzed for luciferase activity using the instructions
and reagents of the Luciferase Assay System, Promega. It was
commonly observed that 2.5×10^{10} viral particles with 6 mg of
plasmid DNA produced the optimal expression of the reporter gene.

New Adenovirus by Cells Infected with Adenovirus and Simultaneously Transduced with the Replication-Enabling Plasmid

Cells infected with an E1A-defective virus and simultaneously transduced with an E1A-containing plasmid in trans resulted in the production of new virus by the infected cells. The pE1A plasmid was complexed to the exterior of the polylysine-conjugated AdCMVlacZ using the optimized viral particle numbers, plasmid amounts, and methods as described above. As a control in these experiments, the polylysine-conjugated AdCMVlacZ was complexed with the pUC 13 plasmid that did not contain any adenoviral sequences. The complexes were applied to the human prostate adenocarcinoma cell line, PC-3, that had been plated at a density of $1.5\text{-}2.0 \times 10^5$ cells/35 mm plate. Each plate received 1/10 of a complex in quadruplicate under conditions described in the previous section. Two hrs later, the plates were washed thrice with phosphate buffered saline and regular medium applied. Two, three and four days later, supernatants and cell lysates were collected. The supernatants were prepared by aspirating the media from the plates for each group, clarifying by centrifugation (1500 RPM at 4C for 5 min) and 0.45 μ m filtration, after which the media was stored at -70C. The lysates were prepared by scraping the plates, then pooling the cells from each group in a total volume of between 0.5 and 0.7 ml regular media. The cells were exposed to four freeze-thaw cycles, the supernatant clarified by centrifugation (3000 RPM in microfuge for 5 min. at 4C) and frozen at -70C.

The supernatants and lysates were tested for the presence of new adenovirus by exposure to 293 cells. If new replication-defective virus was present, the 293 cells should develop the classic cytopathic effect (CPE) of rounding and detachment from the culture dish surface. Figure 3 is a graphic representation of the observed cytopathic effects produced by lysates or supernatants from cells cotransduced with AdCMVlacZ and plasmid DNA. The pUC 13 groups were observed up to 96 hrs after supernatant or lysate addition without any cytopathic effect. The lysates and supernatants from the cells that had been exposed to the AdCMVlacZ + pE1A all produced CPE in the 293 cells, although the onset of CPE was faster with the lysates than the supernatants at all harvest times. In contrast, none

of the lysates or supernatants from the cells exposed to the AdCMVlacZ + pUC 13 developed any signs of CPE. Similar experiments using the human ovarian carcinoma cell line, SK-OV-3 obtained from ATCC also yielded supernatants and lysates from the pE1A groups only that produced CPE in 293 cells.

As further evidence that new virus had been made, adenovirus was recovered from the AdCMVlacZ + pE1A-treated cells and shown to be identical to the starting virus by DNA analysis. Supernatants were collected, clarified by centrifugation, and pooled from the 293 cells that developed CPE following lysate or supernatant exposure (from the virus + pE1A-treated groups). The supernatant pool was applied to confluent plates of fresh 293 cells; each 10 cm diameter dish received 0.5 ml of supernatant in 4 ml of 2% fetal calf serum media. The remainder of the adenovirus amplification was performed as described above. Following the second cesium chloride gradient, the viral band was aspirated and exhaustively dialyzed against 10 mM Tris pH 8.0 at 4°C. The virus was added to an equal volume of 20 mM Tris pH 7.8, 10 mM EDTA pH 8.0, 1.0% SDS containing proteinase K at 100 mg/ml, then incubated at 37°C for between 2 and 16 hrs. The mixture was extracted once with an equal volume of buffered phenol, and DNA precipitated from the remaining solution with ethanol and sodium acetate. The same steps were repeated using the AdCMVlacZ viral stock in order to provide the parent adenoviral DNA specimen for comparison. The DNA pellets were resuspended in 10 mM Trs pH 8.0, 1 mM EDTA and concentration determined by UV spectroscopy (A_{260}/A_{280}). Aliquots (2 mg) of each specimen were cut with restriction endonucleases *Xho* I and *Sal* I, the fragments size fractionated on 1% agarose and the resulting bands compared for relative size. An ethidium bromide-stained photograph of the gel showed identical fragments were produced by the two viral DNA specimens: adenoviral DNA obtained from PC-3 cells following cotransduction with AdCMVlacZ and pE1A and adenovirus made by directly amplifying AdCMVlacZ viral stock. Specifically, the *Xho*I digest produced visualized fragments of approximately 1.4, 2.4, 2.5, 4.3, 5.0, and 15-20 kb; and the *Sal*II digest produced fragments of approximately 25 and 7 kb. This analysis showed that the starting AdCMVlacZ virus, and that produced by the infection of PC-3 cells in the presence of pE1A, yielded similar

fragments demonstrating that the cotransduction of pE1A and AdCMVlacZ resulted in the production of new, replication defective adenovirus, AdCMVlacZ.

5

EXAMPLE 2

Creation of Syngeneic Recombinant Virus-Producing Cells by Cotransduction of Trans-Complementing Plasmid Constructs

10 Recombinant retrovirus is conventionally made by passing a retroviral vector through a "packaging cell line" that has been stably transfected with complementing viral protein coding genes. The sequences of the vector and the packaging sequences are designed so that only the recombinant viral genome will be packaged into new viral particles. The result is an infectious particle that
15 cannot replicate new virus. One widely used retroviral packaging cell line is PA317 that was made by transfecting mouse fibroblasts with the pPAM3 plasmid (Miller 1986).

In order to demonstrate that a plasmid containing the genes required for replication obtained from a packaging cell line and retroviral vector could be co-delivered with the resulting production
20 of new recombinant retrovirus, the appropriate plasmids were cotransduced. The pPAM3 plasmid is described by Miller 1986. A representative retroviral vector, pBAG, was obtained from C.Cepko (Harvard University, Cambridge, MA) that contained a neomycin
25 resistance gene as a selectable marker and a bacterial lac Z gene as described by Price 1987. Figure 4A is a map of pBAG. Figure 4B is a schematic of retroviral sequences within the pPAM3 plasmid.

The plasmids were introduced into cells by ionic attachment to the exterior of an adenovirus. The human type 5
30 adenovirus, DL1014 (Bridge 1989), was modified to contain polylysine on the exterior using the techniques described in Example 1. The pPAM3 and pBAG plasmids were mixed in varying proportions: 1 mg pBAG + 5 mg pPAM3, 3 mg pBAG + 3 mg pPAM3, 5 mg pBAG + 1 mg pPAM3, and 6 mg pBAG alone, in a total volume of 250 ml HBS and
35 added to 2.5×10^{10} polylysine-conjugated viral particles. After incubation at room temperature for 30 min, free polylysine was added as described in example 1. A549 cells or PC-3 cells (both at densities of $1.5\text{-}2.0 \times 10^5/35$ mm plate) ATCC received 1/10 of each

complex in triplicate to quadruplicate for 2 hrs, after which the media was changed to regular medium and the cells grown under usual conditions. Three days post-transduction with the two plasmids, supernatant was aspirated from the cells, clarified by centrifugation
5 followed by 0.45 mM filtration and frozen at -70C until further testing.

The presence of recombinant retrovirus in the supernatant was demonstrated by standard titer assays. Briefly, the supernatant was diluted by log 10 in regular media supplemented with polybrene
10 at 5 mg/ml. The diluted supernatant was applied to A549 cells (5×10^5 cells/60 mm plate) overnight, after which the media was changed to regular media. One day following the media change, the A549 cells were enzymatically detached with trypsin and 1/10 of the cells plated in a fresh culture dish in media supplemented with G418 1 mg/ml.
15 Only cells containing the neomycin resistance gene supplied by the recombinant retrovirus can survive in the G418 media. The result after 10 to 14 days was the appearance of G418-resistant colonies of cells, each colony representing the progeny of a single resistant cell. Figure 5 is a graphic representation of the titers of retrovirus in
20 supernatants of cells cotransduced with pBAG and pPAM3. Supernatants were collected 72 hrs post-transduction with the complementary retroviral vectors. In the supernatant obtained from the cotransduced A549 cells, titers were highest in the groups that received 1 mg pBAG + 5 mg pPAM3 at 4×10^2 /ml. A similar pattern
25 was observed in PC-3 cells that were cotransduced with varying proportions of the complementing plasmids, resulting in titers of 1×10^3 /ml.

As further evidence that new recombinant retrovirus had been made, resistant colonies were shown to express the lac Z gene
30 product. The colonies were washed thrice with phosphate buffered saline, fixed 10 minutes at 4C in 0.2% glutaraldehyde, 50 mM sodium phosphate buffer pH 7.3. Following the fixation period, the plates were washed thrice with phosphate buffered saline, then incubated with the lac Z protein substrate, 5-bromo-4-chloro-3-indolyl-b-D-
35 galactopyranoside (X-Gal) in "X-Gal Solution" (80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 1.3 mM MgCl_2 , 1 mg/ml X-Gal, 3 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 3 mM $\text{K}_4\text{Fe}(\text{CN})_6$) at 37C overnight. Cells containing the lac Z protein turn blue after this treatment. Most of the colonies were stained deeply

blue, indicating the presence of the lac Z product provided by the recombinant retrovirus.

The present invention has demonstrated that multiple cell types can be converted into E1-defective, adenovirus-producing cells by the codelivery of a plasmid encoding the deleted E1 functions and the E1-defective adenovirus. Since this method of replication-enabling the otherwise replication-defective virus is dependent on the presence of the E1-encoding plasmids, this approach is designated Conditional Replication Enablement System for Adenovirus (CRESA). The primary rationale for the development of the CRESA has been as a means of amplifying adenoviral-mediated gene transfer *in vivo*.

The initial CRESA methodology had two aspects that may have mitigated possible clinical utility as a gene transfer amplification strategy. First, the co-delivery of virus and plasmid was achieved by a molecular conjugate comprised of the E1-defective adenovirus conjugated to polylysine ionically linked to the plasmid DNA using methods.

Any requirement for this specific molecular conjugate technology is potentially disadvantageous because of its limited utility *in vivo* and complexity of manufacture. Second, and perhaps more concerning, was the contamination of new virus with small amounts of replication-competent adenovirus (RCA). Although the danger posed by the small amounts of RCA in clinical settings is not clear, clinical studies will be facilitated by a CRESA that does not produce any RCA.

The present invention further shows that CRESA can be achieved by codelivering the replication-enabling plasmids and replication-defective adenovirus to cells by cationic lipid rather than the original molecular conjugate methodology. In addition, alternative plasmids were developed and shown to stimulate the production of new replication-defective adenovirus in the absence of any detectable RCA.

EXAMPLE 3

Cell Lines and Viruses

Cell lines 293 and HeLa were obtained directly from ATCC and maintained in media at atmospheric and temperature conditions. The E1-defective adenovirus containing a firefly luciferase expression

cassette, AdCMV-luc, was provided by R. Gerard (U.T. Southwestern, Dallas, TX). The E1-defective adenovirus containing a herpes simplex virus thymidine kinase expression cassette, AdCMV-HSVTK, was provided by D. Curiel (University of Alabama at Birmingham, Birmingham, AL). The replication-competent virus, wt300, has been described by T. Shenk and was provided by D. Curiel.

EXAMPLE 4

10 Replication-Enabling Plasmids

Plasmids that contained the E1A or E1B gene under transcriptional control of the CMV promoter/enhancer, pCMV-E1A and pCMV-E1B respectively, were provided by E. White (Rutgers University, Piscataway, NJ). The plasmid containing the adenovirus type 5 E1A and E1B genes, pE1, was identical to the plasmid originally designated pE1A.

Plasmids were constructed that contained the E1A and E1B gene regions under transcriptional control of the normal viral elements. The adenoviral inserts for the plasmids were polymerase chain reaction (PCR)-amplified products of the desired gene regions in wt300 DNA. The E1A plasmid, pUC-E1A, contained adenovirus type 5 nucleotides 30 to 1643. These sequences were amplified using the primers Ad5-30F (5' ACT GAA TTC TGA AGC CAA TAT GAT AAT GAG 3') and Ad5-1645R (5' CTC ACT GGA GCG CCA TGC AAG TTA AAC ATT 3') that yielded a product of 1625 bp defined 5' by an Eco RI site and 3' by a Xho I site. The amplified fragment was subcloned into EcoRI/Xho I sites of pSL1180 (Pharmacia, Piscataway, NJ), the resulting plasmid linearized with Hind III and blunted, the adenoviral sequences excised with Eco RI followed by subcloning of the blunt ended/Eco RI fragment into the Eco RI/Sma I sites of pUC 13 by standard techniques. The E1B plasmid, pUC-E1B, contained adenovirus type 5 nucleotides 1623 to 4164. These sequences were amplified using the primers Ad5-1623F (5' ACT GGA TCC GAT AAT GTT TAA CTT GCA TGG 3') and Ad5-4164R (5' CTC ACT CGA GAA AAA ATA CAC AGG ACC CTC 3') that yielded a product of 2551 bp defined 5' by a Bam HI site and 3' by a Xho I site. The amplified fragment was cloned into the Bam HI and Xho I sites of pUC-E1A in order to produce the plasmid pE1-FR. The plasmid pUC-E1B was produced by

excising the E1A fragment from pE1-FR, blunting the overhanging ends and religating by standard techniques.

EXAMPLE 5

5

Cotransduction of Adenovirus and Plasmids

The general protocol simultaneously exposed HeLa cells to both the free, E1-defective adenovirus and plasmid/lipid complexes, a process referred to as "cotransduction". HeLa cells were plated in 24 well plates, 5×10^4 cells/well the evening prior to cotransduction. Plasmids were complexed with a 50%/50% mixture of dioleoyl 1,2-diacyl-3-trimethylammonium-propane/L-dioleoylphosphatidyl ethanolamine (DOTAP/DOPE) (Avanti Polar Lipids, Alabaster, AL) using a lipid:DNA ratio of 2:1 in OPTIMEM (GIBCO/BRL, Gaithersburg, MD) in a volume of 100 l per well. The E1-defective viruses were diluted in OPTIMEM to achieve the desired multiplicity of infection (moi) using the plaque forming unit (PFU) titer for viral number, and the number of HeLa cells originally plated, so that the virus was in a volume of 250 l per well. Cells were washed once with phosphate buffered saline (PBS), the virus added to each well immediately followed by the addition of the lipid/DNA. All groups were performed in at least quadruplicate. The cells were incubated in normal tissue culture conditions for 4 hrs, after which the media was aspirated, the cells washed once with PBS, and fresh growth media added. The cells were maintained in normal tissue culture atmospheric and temperature conditions for 48 hrs, at which time the media was aspirated and replaced with 10% FCS media (250 l/well), and the plates stored at -70° C.

In order to avoid the confounding variables of possible nonspecific toxicity from variable amounts of DNA and cationic lipid, the plasmid dose experiments described by Figure 6b were performed using a constant, total plasmid DNA dose of 1 g per well of the 24 well plate. In these experiments, the constant DNA amount was achieved by using pUC 13 as a "stuffer" in groups that received less than 1 g of pE1.

The experiments that quantified virus present in lysates transduced with the CMV-directed E1A and E1B plasmids were performed with equimolar amounts of the active plasmids. Each well

received the following amounts of plasmid: pE1-0.5 g, pCMV-E1A-0.4 g, pCMV-E1B- 0.45 g in addition to pUC 13 in order to achieve a total DNA dose per well of 1 g. The E1-defective virus was added at an moi of 1, and the remainder of the cotransduction, lysate harvest and plaque analysis done as described above.

The experiments that assessed the influence of the different ratios of pUC-E1A to pUC-E1B used a constant amount of total DNA and adenovirus in all groups. The adenovirus was added at an moi of 1, and the cationic lipid/DNA complexes added in an amount to achieve 1 g plasmid DNA per well using the methods described above. The molar ratios used are specified, all groups were performed in quadruplicate. Adenovirus was quantified in the cotransduced cell lysates by 293 plaque assays as specified above.

15

EXAMPLE 6

Quantification of New Virus Production in Unamplified Cell Lysates

Lysates from cotransduced cells were analyzed for new virus production by plaque assays. The cells were taken through 3 freeze-thaw cycles, the lysate from each group pooled and clarified by centrifugation. The previously described 293 plaque assay was modified to allow 2 days of 293 cell growth to achieve an 80-90% confluence following the initial plating prior to sample exposure, and the cells were overlaid with the molten agar immediately following the 4 hr sample exposure. For the 293 titers, the lowest dilution assayed was 10^{-1} for the negative controls and 10^{-3} for the groups receiving E1A and E1B vectors. The previously described HeLa plaque assay was modified by immediately overlaying the cells after sample exposure with molten agarose (0.8% low melting point agarose, 2% FCS, 25 mM $MgCl_2$, antibiotics in DMEM/F12). For the HeLa titers, the lowest dilution assayed was 1:2 for all groups.

EXAMPLE 7

Quantification of Amplified Replication-Competent Adenovirus

Replication-competent adenovirus was amplified in selected lysates by a two-step assay. In the first step, HeLa cells ("primary cells") were exposed to experimental lysates under the

same conditions as the HeLa plaque assays. Following the 4 hr exposure, the lysate was aspirated and replaced with regular growth media, and the cells grown under normal conditions for 3 days to provide any RCA present to undergo replication. Following the 3 day
5 replication period, lysates were harvested from the primary cells and exposed to A549 cells under conditions identical to the standard HeLa plaque assay. Plaques were quantified 7 days later.

Lipid-mediated codelivery of the pE1 plasmid and E1-defective adenovirus resulted in the production of new, E1-defective
10 adenovirus. The multiplicity of infection (moi) of the initial E1-defective (AdCMV-luc) virus exposure was shown to significantly modulate the amount of new E1-defective virus produced (Figure 6A). In these experiments, a fixed amount of plasmid was added to each well (1 g) as the moi was varied from 0.1 to 10. At the moi of 0.1, the
15 cells exposed to pE1 and AdCMV-luc produced 0.8×10^6 PFU/ml lysate of adenovirus detected by 293 cell plaque assays. At the same moi, the controls that were cotransduced with the same virus and the pUC control plasmid had less than 10 recombinant PFU. At moi's of 1 and 10, the amounts of virus increased to 9.1 and 30.0×10^6
20 recombinant PFU/ml lysate, respectively. The amount of recombinant virus produced by the pUC controls at moi's 1 and 10 increased to 3.5 and 24.2×10^1 PFU, respectively. The amount of new E1-defective virus produced also varied with the amount of replication-enabling plasmid added to the cells (Figure 6B). In these experiments, the moi
25 was fixed at 1 as the amount of plasmid added ranged from 0.01 to 1 g per well. In the range of 0.05 to 1 g, the values ranged from 1.1 to 5.4×10^6 PFU/ml lysate of recombinant virus with a statistically insignificant trend towards incrementally higher titers as the plasmid amounts were increased. These results show that new recombinant
30 virus could be produced by codelivery of E1-defective virus and the replication-enabling plasmid using cationic lipid rather than the molecular conjugate as the means of facilitating entry of the pE1 plasmid.

The cationic lipid-mediated delivery of the pE1 plasmid in
35 combination with the E1-defective virus did not eliminate the problem of contaminating, RCA production (Figure 7). In multiple experiments, lysates from cells cotransduced with the pE1 plasmid and AdCMV-luc produced small amounts of adenovirus detectable by

HeLa plaque assays, i.e., RCA. In these experiments (n=3), an average of 5.9 1.4 HeLa plaques were present per ml of lysate that had an average 293 adenoviral titer as determined by 293 plaque assays of 3.4 0.8 x 10⁶. In agreement with experiments using the molecular conjugate delivery system, the groups that received pUC 13 and AdCMV-luc did not produce any virus detectable by the HeLa plaque assays. Thus, the majority of the viral plaques measured by the 293 plaque assays were replication-defective, but that 1.7 per 10⁶ 293 plaques were RCA as determined by the HeLa plaque assays. Thus, the change from molecular conjugate to cationic lipid-facilitated delivery of the pE1 plasmid did not overcome the problem of RCA contamination in this codelivery system.

As a first step towards redesigning the replication-enabling plasmid as a means of overcoming the replication-competent adenovirus contamination problem, CMV-directed E1A and E1B coding sequences on separate plasmids were compared to the original pE1 plasmid (Figure 8). Equimolar amounts of the CMV-directed E1A and E1B plasmids, or the pE1 plasmid, were cotransduced with the AdCMV-luc virus and new virus quantified in lysates by 293 plaque assays. Groups that received the pUC control, or the pCMV-E1B alone, produced measurable but minimal amounts of adenovirus. The pCMV-E1A alone yielded a mean of 1.6 x 10³ PFU per ml lysate that was more than a log greater than that produced by the pCMV-E1B alone. The combination of the pCMV-E1A and pCMV-E1B plasmids produced a mean of 1.3 x 10⁴ PFU per ml lysate, but this amount of virus was much less than the mean of 3.7 x 10⁶ PFU per ml lysate that was observed with the pE1 plasmid in this series of experiments. Although the combination of the E1A and E1B plasmids yielded a synergistic increase in the number of viruses produced compared to either plasmid alone, the total amount was over 2 logs lower than that achieved with an equimolar amount of the pE1 plasmid. Although replication competent adenovirus was not detected in the lysates of cells cotransduced with pCMV-E1A + pCMV-E1B (not shown), the total number of viruses produced was so low that low frequency RCA production would not have been detected by these determinations.

New replication-enabling plasmids were developed with the intent of maintaining a level of recombinant virus production comparable to the pE1 plasmid while eliminating RCA production

(Figure 9). The encoding sequences were produced by PCR amplification of the E1A and E1B encoding sequences in wt300 DNA. The amplified sequences of each region were designed to encompass the native promoter and enhancer regions, and were bracketed by new restriction sites added by the PCR. Northern blot analysis of total cellular RNA prepared from HeLa cells transduced with pUC-E1A or pUC-E1B demonstrated that they produced E1A and E1B transcripts that co-migrated with those present in 293 cell RNA specimens.

Cotransduction of the new replication-enabling plasmids with the E1-defective virus resulted in the production of new virus in amounts comparable to that achieved with the original pE1 plasmid (Figure 10). The pUC-E1A and pUC-E1B plasmids were administered using a range of E1A:E1B ratios while keeping the total amount of plasmid added to the cells constant. The amounts of virus produced by the pE1 and pUC 13 groups, respectively the positive and negative controls, were in agreement with the previous results. The administration of pUC-E1A alone with the virus yielded a mean of 2.2×10^4 PFU/ml in the three experiments. In contrast, the pUC-E1B alone yielded a mean of 22 PFU/ml. Exposure of cells to the E1-defective virus and the mixtures of pUC-E1A and pUC-E1B over a wide range of ratios resulted in a marked, synergistic increase in new virus production. The ratios (E1A:E1B) ranging from 5:1 to 1:10 all resulted in $1-5 \times 10^6$ PFU per ml of lysate without a statistically significant difference between the groups.

A sensitive assay for the detection of replication-competent adenovirus (RCA) identified conditions that eliminated all detectable RCA (Figure 11). Initial, conventional plaque assays using HeLa as the indicator cell line appeared to show that the pUC-E1A+pUC-E1B combination had eliminated most, if not all RCA (not shown). However, at the highest concentrations of lysate exposure (1:2 dilution), multiple small areas of possible viral cytopathic effect (CPE) were observed. Many attempts to propagate additional virus from these small areas of CPE were unsuccessful. As a more stringent test for RCA, an amplification step was added as a means of favoring growth of RCA in the absence of contaminating replication-defective virus that may have been obscuring the results at the most concentrated lysate samples tested with the conventional plaque assays. In these experiments, the lysates from HeLa cells

cotransduced with the E1-defective virus and the various replication-enabling or control plasmids were harvested and exposed to HeLa cells as if for a conventional plaque assay. However, following the lysate exposure, the cells were grown an additional 3 days that was expected to provide opportunity for any RCA present to be amplified within the HeLa cells. These secondary lysates were then exposed to A549 cells as a means of detecting RCA. The addition of 5 wt 300 virus PFU's to the primary HeLa plates resulted in more than 10^4 PFU on the secondary A549 plates. In the typical experiment shown here, very small amounts of RCA were present in the lysates from cells exposed to the higher amount of plasmid (10 g/10 cm plate) that was 2 logs less than the amount present in the lysates of the cells exposed to pE1. However, reducing the amount of plasmid cotransduced to 1 g per plate eliminated all detectable RCA in the 1:2 and 1:10 ratio groups, although 2 plaques were detected in this experiment with the 5:1 ratio. At this lower amount of plasmid, the amount of adenovirus detectable by the 293 plaque assays did not significantly decrease. These experiments showed that very small amounts of RCA could be detected by this amplification strategy even when the E1A and E1B functions were administered on separate plasmids under certain conditions. Thus, the major determinant of the RCA was excess plasmid which could be eliminated by using conditions whereby plasmid was administered in lower amounts.

The original molecular conjugate system was effective in demonstrating some of the basic concepts of the conditional replication enablement system for adenovirus (CRESA). The molecular conjugate system employed demonstrated significant lot-to-lot variability of the adenovirus-polylysine conjugate in the context of CRESA efficacy, and this problem made the relatively short shelf life of these conjugates a major disadvantage. In addition, anecdotal reports that the specific molecular conjugate system employed did not work well *in vivo* was also of concern. The present invention demonstrated that the CRESA is not dependent on the molecular conjugate means of codelivery, since new E1-defective adenovirus was produced by cells transduced with cationic lipid-complexed, E1-encoding plasmids and free adenovirus. Although the comprehensive experiments shown here were performed with a single cell line, the

same methodology has been applied successfully to multiple lung and ovarian carcinoma cell lines (not shown).

Small amounts of contaminating, RCA were found in cells cotransduced with the original replication-enabling plasmid (pE1) and the E1-defective virus by cationic lipid methods. In these experiments, that utilized the original pE1 plasmid for replication-enablement, RCA was produced at a frequency one order of magnitude less than with the original molecular conjugate delivery. No RCA was produced by the control groups that were cotransduced with the E1-defective adenovirus and control plasmid not containing any viral sequences. It was concluded that homologous recombination between the adenoviral sequences in pE1 and the E1-defective adenovirus was the most likely cause of the RCA.

It was expected that separating the E1A and E1B functions on separate plasmids would eliminate the RCA by requiring two homologous recombination events in order to restore the E1A and E1B functions. The CMV-directed E1A and E1B constructs did not produce any detectable RCA, but the total amount of virus produced was so small that it was not apparent that the problem had been overcome by this strategy. The reason for the much lower viral titer achieved by the CMV-directed plasmids compared to the original pE1 plasmid was not determined. Since the pUC-E1A and pUC-E1B plasmids did replication-enable to an extent comparable to the pE1 plasmid, the separation of the E1A and E1B functions with the CMV-directed plasmids per se did not explain the findings. Experiments were also performed demonstrating that the CMV-directed plasmids were not directly toxic to the cells as assessed by cell growth assays.

The plasmids disclosed by the present invention in which the native viral promoter/enhancer regions were preserved for E1A and the promoter region preserved for E1B were effective in mediating the production of large amounts of new replication-defective virus. In agreement with the CMV-directed plasmid results, the E1A plasmid alone mediated the production of significantly more adenovirus than the E1B plasmid alone. The combination of the E1A and E1B plasmids resulted in a much greater increase in virus production than was observed by the combination of the CMV-directed plasmids. The relative proportions of the E1A and E1B plasmids did not appear to be a critical determinant of new virus

production, since a broad range of E1A and E1B mixtures did not result in major differences in new virus production.

Under conditions of plasmid excess, minimal amounts of RCA consistently detectable only with an RCA amplification step were produced in association with replication-enablement by the pUC-E1A and pUC-E1B plasmids. The genetic structure of the few RCA recovered were not determined, but it is reasonable that a dual recombination event occurred in which both E1A and E1B functions were reincorporated into the E1-defective adenoviral genomic backbone. The frequency was not significantly changed by altering the ratio. Importantly, all detectable RCA was eliminated by simply reducing the amount of plasmid by an amount that did not significantly change the total amount of virus produced. In conclusion, CRESA can be accomplished by at least two means of codelivering replication-defective virus and replication-enabling plasmid. New replication-enabling vectors and the appropriate conditions for their use have been described that eliminates all detectable RCA.

EXAMPLE 8

In Vitro Amplification of HSVTK Mediated Toxicity

The cationic lipid mediated delivery of the replication enabling plasmids for CRESA was shown to amplify response to a therapeutic gene carried by a replication defective adenovirus (Figure 12). For these experiments, the adenovirus was AdCMVHSV tk, provided by David Curiel, in which the herpes simplex virus thymidine kinase coding sequence is under transcriptional control of the CMV promoter enhancer. Cells containing the HSVTK protein as a consequence of infection with this virus are killed by exposure to the antiviral agent, ganciclovir. The replication enabling plasmid used was pE1. HeLa cells were cotransduced with the pE1 (controls pUC 13) and AdCMVHSV tk under conditions identical to those described above. One day later, the cells were enzymatically detached, enumerated, and mixed with increasing percentages of "naive" (unmanipulated) HeLa cells for a 4 day coculture period, after which the cells were replated at a fixed amount in media containing 20 mM ganciclovir. The results demonstrated that at all the different

mixtures of cotransduced + naive cells, those groups receiving the pE1 plasmid had a markedly increased level of killing compared to the control groups that received the same amount of AdCMVHSV-tk and the pUC plasmid. This data provided *in vitro* evidence that the CRESA approach could achieve amplification of a desired therapeutic response to a gene carried by a replication defective adenovirus.

EXAMPLE 9

In Vivo Amplification of HSVTK Mediated Toxicity

The cationic lipid mediated delivery of the replication enabling plasmids for CRESA was shown to amplify response to the AdCMVHSV-tk virus *in vivo* (Figure 13). In these experiments, the replication enabling plasmids were a mixture of pSVN20 (encoding the 13S E1A protein as described by Smith et al., Mol. Cell. Biol. 5:2684 (1985) and pUC E1B. A549 cells were cotransduced with these two plasmids, or pUC 13 in control groups, and the AdCMVHSV-tk virus as described above. One day later, the cells were detached, enumerated, and mixed in a 1:5, and 2:5 ratio with naive A549 cells. The cell mixtures were mixed with Matrigel, and 2×10^6 cells engrafted by subcutaneous injection into the flanks of athymic nude mice. The right flank of each mouse received the mixture that had been exposed to the replication-enabling plasmids, and the left flank the mixture exposed to the pUC control. At 4 days after engraftment, the mice were treated by b.i.d. intraperitoneal injections of ganciclovir at a dose of 75 mg/kg for a total of 7 days. Mice were sacrificed 15 days after engraftment, and the tumors weighed. This experiment demonstrated that the tumors containing the replication enabled AdCMVHSV-tk were two to three fold smaller than the control tumors treated with the same amount of AdCMVHSV-tk and the control plasmid (Figure 13). This is evidence that the CRESA methodology can be used to augment a therapeutic response *in vivo*.

EXAMPLE 10

35

RNA Vectors for Directing Synthesis of Replication-Enabling Proteins

RNA vectors encoding the E1 trans activating proteins were constructed and cotransduced with E1-defective adenovirus as

an alternative means of achieving CRESA. The development of these vectors proceeded in two successive steps. First, in order to establish the concept that RNA can be administered in lieu of plasmid DNA for replication enablement in the context of CRESA, the appropriate RNA transcripts were manufactured ex vivo and tested by cationic lipid-mediated administration for the capacity to replication-enable E1-defective adenovirus. Second, several replicative RNA vectors were constructed and tested in the CRESA context to achieve a greater magnitude of new virus production than with the non-replicative RNA transcripts used for the proof of concept experiments. The design of RNA vectors is based on the fact that the RNA vectors are not be capable of recombining with the defective viral DNA genome so that all replication-competent adenovirus production is avoided.

Although the E1A and E1B gene regions encode multiple transcripts, the transcripts essential for activation of viral replication have been well delineated, so that the vectors can be designed for the production of the necessary transcripts. In the case of E1A, the 13S transcript encoding the 289 amino acid protein has been identified as that which is necessary and sufficient for the E1A component of the E1 trans activation. In the case of E1B, the 19 Kd and 52 Kd proteins have both been shown to be important components for the viral replication. Fortunately, both proteins are encoded by a single 2.2 kb E1B transcript via translation of alternative reading frames so that a vector making the single transcript will encode for both proteins.

In order to achieve the first step in the RNA vector development, vectors were constructed to generate large amounts of the relevant transcripts by in vitro transcription. The isolated transcripts were administered to the target cells by cationic lipid-facilitated transduction in conjunction with the administration of the E1-defective adenovirus. The E1A and E1B DNA templates were inserted into the RNA transcription vector, pSP64T. The pSP64T vector is a derivative of pSP64 that is designed to generate transcripts under in vitro conditions that contain 5' and 3' untranslated regions of xenopus globin mRNA as a means of augmenting transcript stability. The template for producing the 13S E1A transcript has been directly derived from the pSVN20 vector. This sequence includes the native E1A promoter region with elimination of intron sequences so as to produce the appropriate 13S coding sequence. This vector has been

tested in the DNA form and shown to appropriately activate replication of E1-defective virus in the presence of E1B encoding plasmids (not shown). The 13S coding sequence was excised with restriction endonucleases, the overhangs blunted, and the sequence
5 ligated to the similarly blunted Bgl II cloning site in the pSP64T to produce the transcription vector pSP64T/289E1A. Transcripts were generated and the RNA clarified by DNAase digestion to eliminate the plasmid DNA followed by phenol-chloroform extraction and ethanol precipitation. In preliminary experiments, these E1A 13S transcripts
10 have been substituted in place of the E1A plasmid vector to achieve a comparable level of new E1- defective adenovirus in the CRESA (Figure 14). These results illustrate that RNA transcripts can replace plasmids as vectors for delivery of the replication-enabling functions in the context of CRESA.

15 Since vectors have not identified that encode the requisite 2.2 kb E1B transcript required for replication-enablement in conjunction with the 13S E1A transcript, it was necessary to construct the appropriate DNA template. The E1B insert in the plasmid pUC-E1B was digested with restriction endonucleases Bgl II and Mun I to
20 remove the intron included between adenovirus serotype 5 nucleotides 3328 and 3924. The in-frame corresponding coding sequences were obtained by excising the Bgl II/Mun I fragment from the cDNA corresponding to the 2.2 kb E1B mRNA that were made by RT-PCR using appropriate primers. The 2.2 kb transcript was
25 identified in total cellular RNA specimens harvested from 293 cells, and was the source of RNA for the RT-PCR. Primers have been designed to amplify a 693 bp fragment of the mRNA corresponding to Ad 5 nucleotides 3287 and 3980. This fragment was cut with restriction endonucleases Bgl II and Mun I, and directly cloned into
30 the corresponding sites of the E1B insert from pUC-E1B cut with the same enzymes. The result was a plasmid that uses the minimal, native E1B promoter to direct the production of the 2.2 kb transcript. This insert was cloned into the pSP64T vector as described for the 13S E1A construct.

35 As the second step in the development of the RNA vectors, replicative RNA vectors that produce the requisite E1A and E1B transcripts were constructed. The Sinbis vectors, TLXN and SinRep5 were supplied by Theresa Strong. The same DNA templates used for

generating the transcripts were inserted into the TLXN and SinRep5 vector at the appropriate cloning site by standard techniques. The Semliki Forest virus vector, pSFV-1 was supplied by Theresa Strong. Again, the DNA templates used for E1A and E1B transcript generation
5 were inserted into the pSFV-1 vector at one of the three unique cloning sites by standard techniques. The poliovirus replicon vector, (pT7-IC), was supplied by Dr. Casey Morrow. As for the previous two replicative vectors, the appropriate DNA templates were cloned into the cloning site of the replicon plasmid, pT7IC.

10

EXAMPLE 11

Demonstration of New Adenovirus Production by Cells Cotransduced with Viral and Plasmid DNAs

15 AdCMVluc viral DNA was prepared. The AdCMVluc viral DNA and pE1A plasmid DNA were cotransduced into HeLa cells using cationic lipid, 1,2-dimyristoyl-3-trimethylammonium-propane (DOTAP), as a facilitator of DNA transfection. The molar ratio of replication-enabling plasmid DNA:viral DNA is a critical variable, as is
20 the ratio of DOTAP to total DNA being introduced to the cells. The molar ration (plasmid:viral) of about 1:1 to 4:1 is necessary in conjunction with a lipid to DNA ratio of 2:1. Unlike the original method of codelivery, where new virus production was evident at 72 hours, no new virus could be detected until 6-8 days post-transduction of the DNAs.

25 The plasmid and viral DNAs were gently mixed together in OPTIMUM™ (GIBCO/BRL) to produce a total DNA concentration of 100 ng/μl. In a separate tube, DOTAP was mixed with OPTIMUM™ to yield a lipid concentration of 200 ng/μl. The DNA tube contents were then
30 added and gently mixed with the DOTAP tube, and the tube incubated at room temperature for 10 minutes. HeLa cells (10⁵ cells/well of a 24 well plate) were washed, and 200 μl of OPTIMUM™ added. After the incubation period, 10 μl of the mixture was added to each well of the HeLa's that were subsequently placed in the usual 37° C
35 humidified/CO₂ atmosphere. Four hours later, the media was aspirated and replaced with DMEM/F12 supplemented with 2% fetal calf serum media. Supernatants were collected 2, 4, 6, and 8 days later and examined for the capacity to transfer luciferase activity to

fresh HeLa cells as evidence of new AdCMVluc production. Luciferase activity was only conferred by supernatants from the cells 6-8 days post transduction as shown in Figure 15.

The replication-enablement technology amplified
5 adenoviral delivered transgene expression within a prostate carcinoma model system. The system was tested by measuring the amount of adenoviral transgene expression, i.e., luciferase activity, within tumors engrafted in nude mice. Tumors were produced by administering the PC-3 cells (1×10^7 cells SQ, flanks) that achieved an
10 8-10 mm diameter in about 4 weeks in athymic nude mice. At that time, each tumor was injected once with 25 μ l of AdCMVluc/pE1A complex so that each tumor received 8.3×10^8 viral particles complexed with 198 ng plasmid DNA. The tumors were excised 10 days later and the lysates made with a polytron on ice and luciferase
15 activities and protein concentration determined. Those tumors transduced with AdCMVluc and pUC-13 (=7) had significantly lower luciferase activity per mg of protein. There was a consistently increased amount of luciferase expression in the tumors that received the virus as shown in Figure 16.

20

EXAMPLE 12

In order to achieve replication-enablement of E1-defective adenovirus with only ribonucleic acids as a means of improving
25 safety, it was necessary to construct a novel E1B vector. The E1B region encodes multiple transcripts produced by variations in splicing, but the 2.2 kb transcript encodes the two proteins that are necessary and sufficient for replication when combined with the E1A 13s transcript product. A DNA template that would produce the 2.2 kb
30 transcript was therefore created. As the first step, the adenoviral DNA corresponding to the adenovirus serotype 5 nucleotides 3287 to 3980 minus the intron defined by nucleotides 3511 to 3594 was obtained by reverse transcriptase-polymerase chain reaction amplification of the 2.2 kb transcript. Total RNA was obtained by
35 routine techniques from 293 cells (American Type Culture Collection, Rockville, MD) as a source of E1 transcripts. Random hexamers were mixed to achieve a concentration of 50 pmoles/microliter with 5 micrograms of 293 RNA in a volume of 12 microliters. The mix was

heated to 70°C for 10 minutes, cooled to 37°C at a rate of 1°C per minute. To this mixture was added 4 microliters of 5X RT buffer, 2 microliters of dNTPs (10 mM), 1 microliter of 100 mM DTT, and 1 microliter of reverse transcriptase, and the mixture incubated at 42 °C
5 for 50 min. to reverse transcribe the DNA copy of the desired region on the RNA transcript. Ten microliters of this product was used as the template for the PCR reaction. Primers 3287F (5' gtc caa ggt gaa cct gaa cg 3') and 3980R (5' cag atc caa cag ctg ctg ag 3') were added to achieve a 100 micromolar concentration, dNTPs added to achieve a
10 200 micromolar concentration, magnesium chloride to achieve a 3 millimolar concentration, tris HCL added to achieve a 20 millimolar concentration, KCL added to achieve a 50 millimolar concentration, and 1.25 units of taq polymerase added in a total volume of 100 microliters. PCR thermocycles included an initial denaturation at 94°C
15 for 2 min followed by 10 cycles of annealing at 60°C, extension at 72°C, and denaturation at 94°C (1 min. for each condition). Fifty microliters of this product was digested with restriction endonuclease Afl II that would cut any undesired product containing the intron sequences, and the resulting digest used as a template for another PCR reaction using
20 the original concentrations of ingredients and identical thermocycles for 30 more cycles.

The 608 bp product was digested with restriction endonucleases Mun I and Bgl II, and the resulting 511 bp fragment gel purified using standard techniques. The Mun I/Bgl II fragment was
25 subcloned into the pUC-E1B plasmid described above that had been linearized with a Mun I/Bgl II digest to generate the new plasmid containing the template for the 2.2 kb E1B transcript that is designated pB2.2. Extensive restriction endonuclease digests confirmed that the intron sequences had been removed.

30 Experiments confirmed that pB2.2 can replication-enable E1-defective adenovirus replication in combination with E1A. A549 cells (American Type Culture Collection, Rockville, MD) at 50,000 cells per well of 24 well plates were cotransduced with the AdCMV-luc adenovirus at a multiplicity of infection of 1 and with the plasmids
35 that were complexed with the cationic lipid DOTAP/DOPE so that each well received 1 microgram total of DNA (0.5 micrograms of each plasmid per group). The mixture was removed in 4 hrs, normal media placed and the cells incubated for 48 hrs under normal tissue culture

conditions, at which time cell lysates were prepared. Adenovirus present in the lysates was quantified by 293 plaque assays to yield the following results: pB2.2 + pUC 13 less than 100 PFU/ml lysate

pB2.2 + pUC-E1A	1,600,000 PFU/ml lysate
pUC-E1B + pUC-E1A	3,500,000 PFU/ml lysate
pUC-E1A + pUC 13	18,000 PFU/ml lysate

These results show that the pB2.2 in combination with the E1A plasmid does replication-enable the E1-defective adenovirus. This sequence were placed into the pSP64T vector to generate RNA transcripts so that replication-enablement can be performed without any E1 DNA sequences which would cause safety problems due to homologous recombination or insertion into the host cell genome.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WE CLAIM:

1. A method for creating recombinant virus-producing cells, comprising:

5 selecting a first nucleic acid sequence comprising a viral genome which is not capable of directing the production of new viral particles in the absence of additional viral nucleic acid sequence;

 constructing a second nucleic acid sequence comprising a viral gene sequence consisting essentially of the viral genes required for
10 the first nucleic acid sequence to produce new viral particles in a cell in which the first nucleic acid sequence is otherwise unable to direct the production of new viral particles, in combination with means for replicating the second nucleic acid sequence; and

 inserting the combination of the first and second nucleic acid
15 sequences into the cell in which the first nucleic acid sequence is unable to direct the production of new viral particles in the absence of the second nucleic acid sequence.

2. The method of claim 1, wherein the first and second nucleic
20 acid sequences are coupled.

3. The method of claim 2, wherein the sequences are coupled covalently.

25 4. The method of claim 2, wherein the sequences are coupled ionically.

5. The method of claim 1, wherein the first and second nucleic acid sequences are inserted by transduction.

30

6. The method of claim 1, wherein the first and second nucleic acid sequences are inserted by means of a virus capable of infecting the cell.

35

7. The method of claim 6, wherein the virus is rendered non-viable prior to infection.

8. The method of claim 1, wherein the first or second nucleic acid sequences further comprise nucleic acid sequence selected from the group consisting of nucleic acid sequence that codes for proteins used to identify cells infected with recombinant virus produced by the combination of the first and second nucleic acid sequences, nucleic acid sequences that codes for proteins that function to kill cells containing the viral genome, nucleic acid sequence that codes for therapeutic proteins that will serve to treat a pathophysiologic condition within the body, and nucleic acid sequence encoding biologically active nucleic acids.

9. The method of claim 8, wherein the first nucleic acid sequences comprising coding nucleic acid sequence.

10. The method of claim 1, wherein the virus is selected from the group consisting of adenoviruses, herpesviruses, adeno-associated viruses, and retroviruses.

11. A composition for creating recombinant virus-producing cells comprising
a first nucleic acid sequence comprising a viral genome which is not capable of directing the production of new viral particles and
a second nucleic acid sequence comprising a viral gene sequence consisting essentially of the genes required for the first nucleic acid sequence to direct the production of new viral particles in a cell in which the first nucleic acid sequence is otherwise unable to direct the production of new viral particles, in combination with means for replicating the second nucleic acid sequence in a cell.

12. The composition of claim 11, further comprising a cell in which the first nucleic acid sequence is unable to direct the production of new viral particles in the absence of the combination of the first and second nucleic acid sequences.

13. The composition of claim 11, wherein the first and second nucleic acid sequences are coupled.

14. The composition of claim 13, wherein the sequences are coupled covalently.

15. The composition of claim 13, wherein the sequences are
5 coupled ionically.

16. The composition of claim 11, further comprising an agent facilitating transduction of a mammalian cell by the first and second nucleic acid sequences.

10

17. The composition of claim 11, wherein the first or second nucleic acid sequences further comprise nucleic acid sequence selected from the group consisting of nucleic acid sequence that codes for proteins used to identify cells infected with recombinant virus
15 produced by the combination of the first and second nucleic acid sequences, nucleic acid sequences that codes for proteins that function to kill cells containing the viral genome, nucleic acid sequence that codes for therapeutic proteins that will serve to treat a pathophysiologic condition within the body, and nucleic acid sequence
20 encoding biologically active nucleic acids.

18. A method for expressing exogenous nucleic acid sequence in a mammalian cell, comprising:

introducing into a cell a first nucleic acid sequence comprising a
25 viral genome which is not capable of directing the production of new viral particles and a second nucleic acid sequence comprising a viral gene sequence consisting essentially of the genes required for the first nucleic acid sequence to direct the production of new viral particles in a cell in which the first nucleic acid sequence is unable to otherwise
30 direct the production of new viral particles in combination with means for replicating the second nucleic acid sequence in a cell, wherein the first or second nucleic acid sequence is selected from the group of nucleic acid sequence consisting of nucleic acid sequence that codes for proteins used to identify cells infected with recombinant
35 virus produced by the combination of the first and second nucleic acid sequences, nucleic acid sequences that codes for proteins that function to kill cells containing the viral genome, nucleic acid sequence that codes for therapeutic proteins that will serve to treat a

pathophysiologic condition within the body, and nucleic acid sequence encoding biologically active nucleic acids.

19. The method of claim 18, wherein the first and second
5 nucleic acid sequences are coupled.

20. The method of claim 19, wherein the sequences are coupled covalently.

10 21. The method of claim 19, wherein the sequences are coupled ionically.

22. The method of claim 18, wherein the cells are in culture.

15 23. The method of claim 18, wherein the cells are in a patient.

24. A method for killing replicating cells, comprising:
administering to the replicating cells an adenovirus which is
unable to direct the production of new viral particles in the
20 replicating cells,

administering to the replicating cells a retrovirus comprising
a viral gene sequence consisting essentially of the genes
required for the adenovirus to direct the production of new viral
particles in the replicating cells in which the adenovirus is otherwise
25 unable to direct the production of new viral particles.

25. The method of claim 24 wherein the replicating cells are tumor cells.

30 26. The method of claim 25 wherein the replicating cells are in the brain.

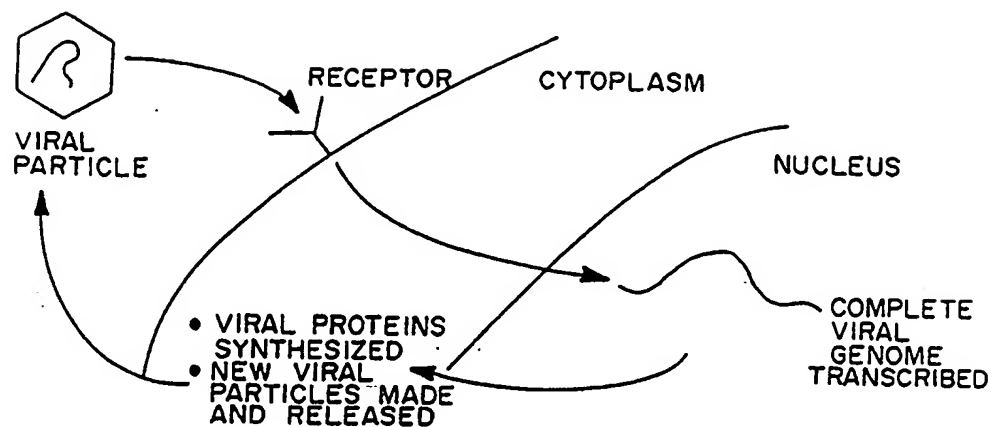
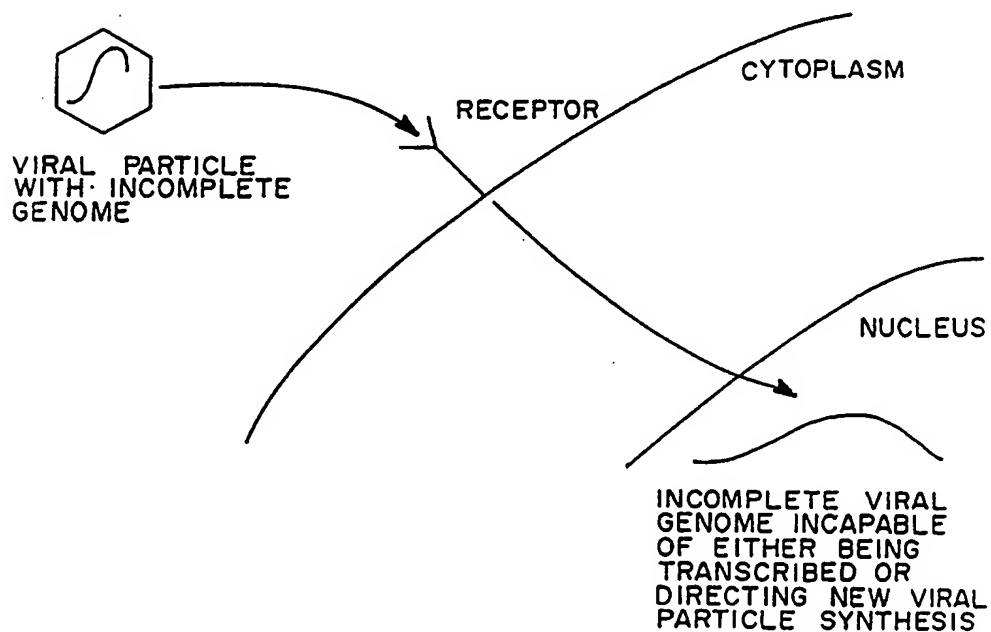
27. A composition for killing replicating cells comprising
an adenovirus which is unable to direct the production of new
35 viral particles in the replicating cells, and

a retrovirus comprising
a viral gene sequence consisting essentially of the genes
required for the adenovirus to direct the production of new viral

particles in the replicating cells in which the adenovirus is otherwise unable to direct the production of new viral particles.

28. The composition of claim 27, wherein the replicating cells
5 are tumor cells, further comprising the cells.

1/15

**FIGURE 1a****FIGURE 1 b**

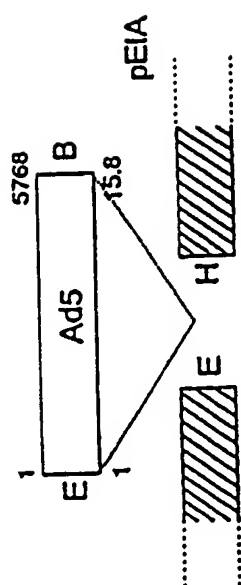


FIGURE 2b

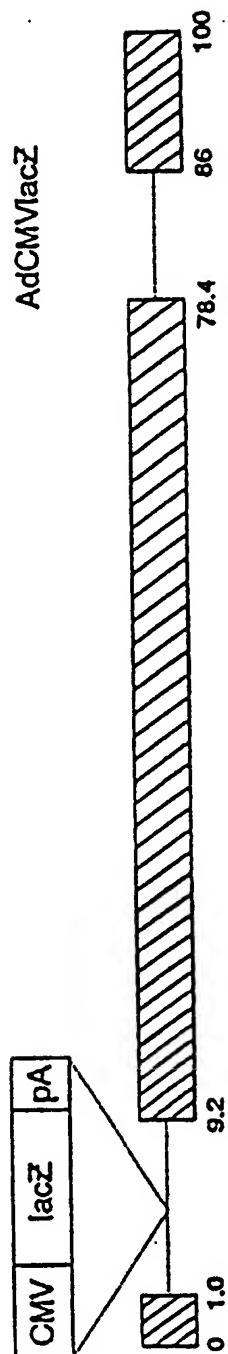


FIGURE 2a

3/15

3/15

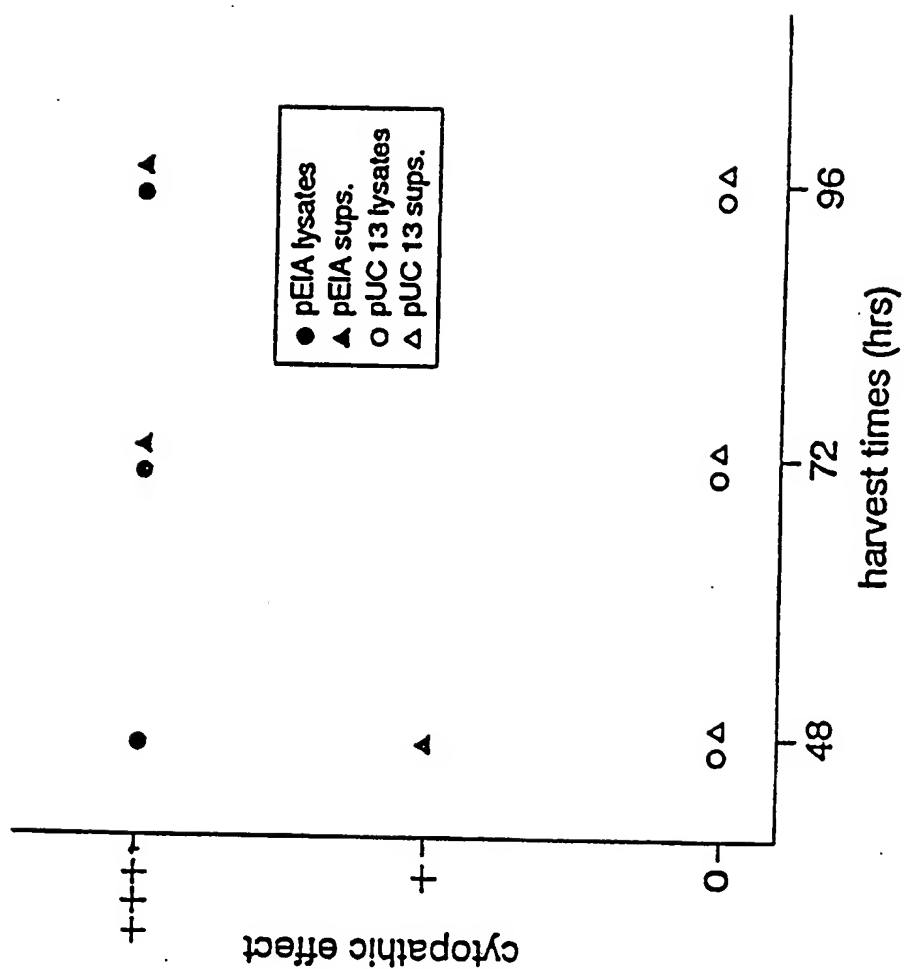


FIGURE 3

4/15

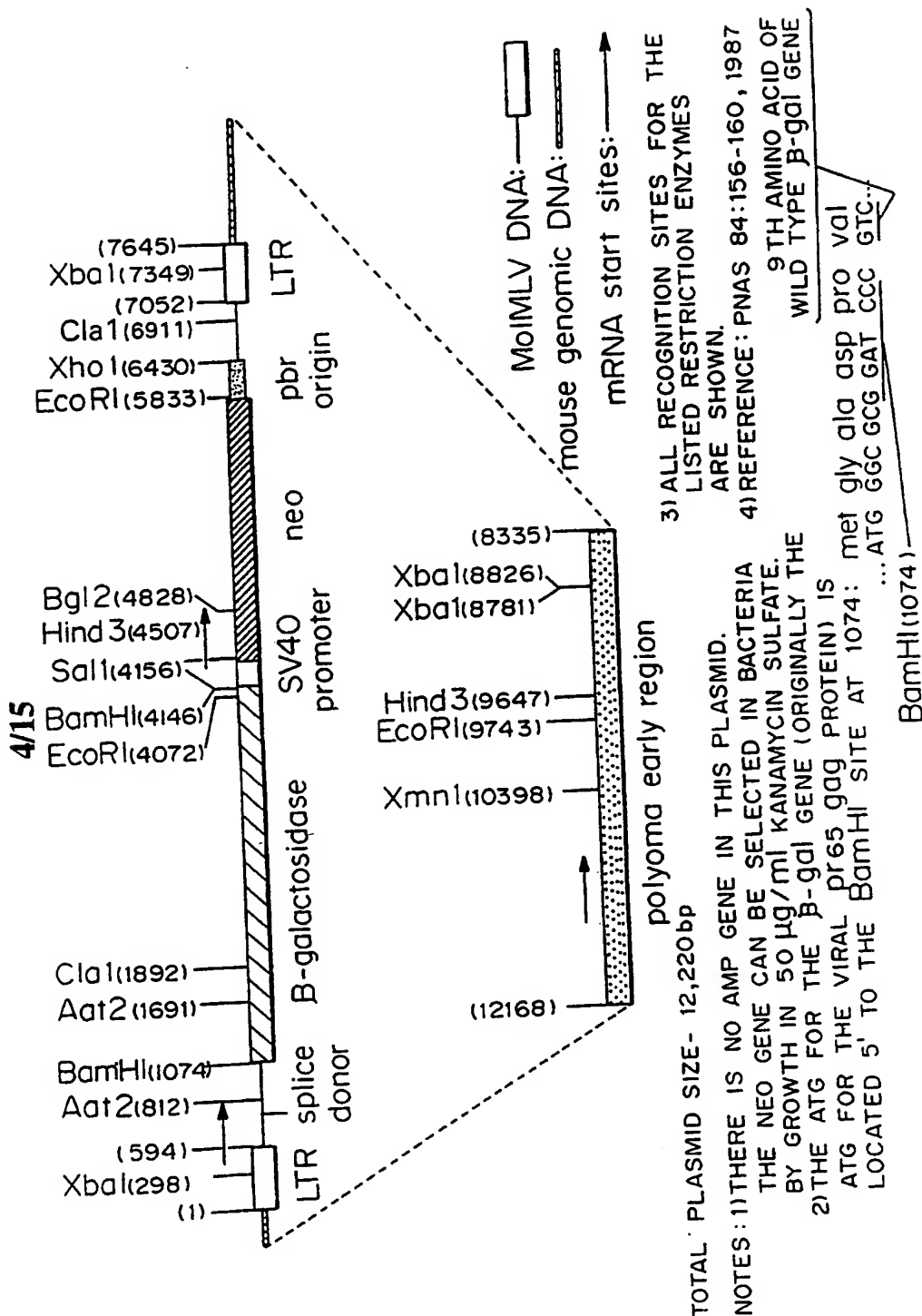


FIGURE 4a

5/15

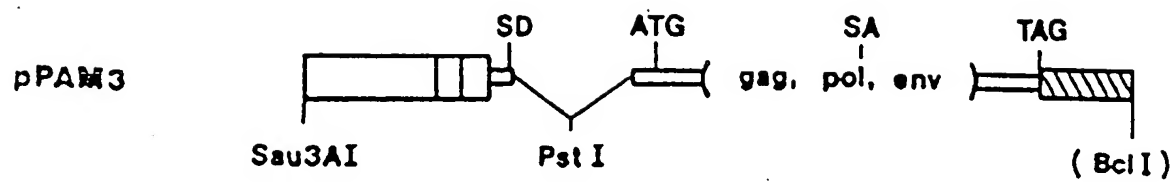


FIGURE 4b

6/15

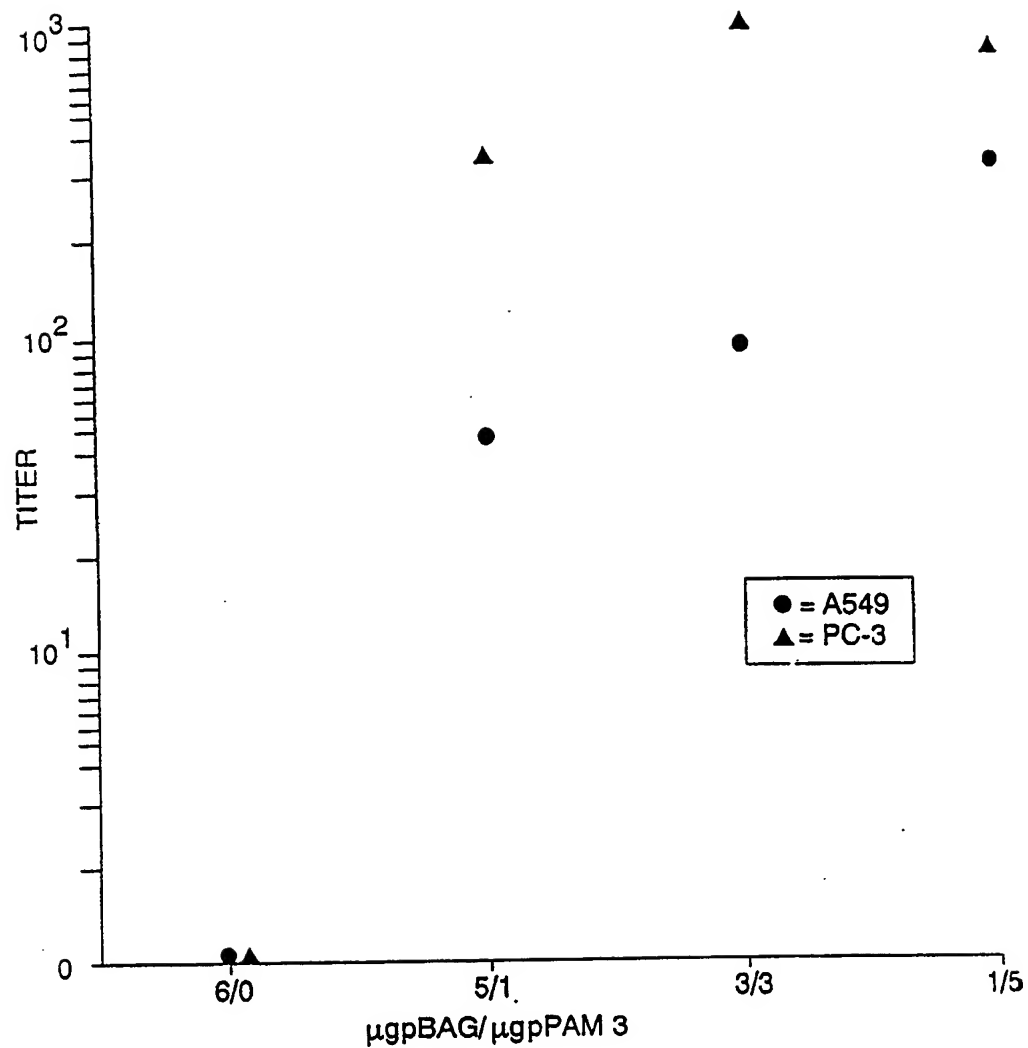


FIGURE 5

7/15

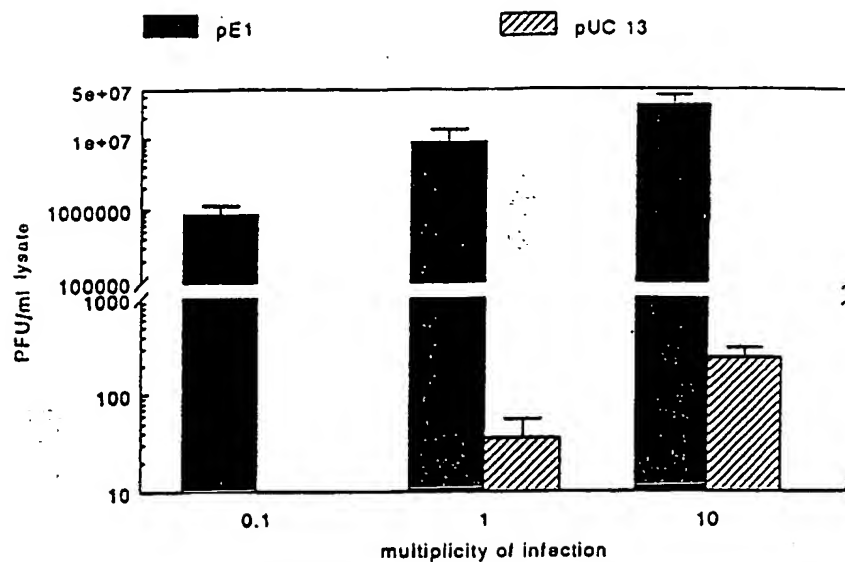


FIGURE 6a

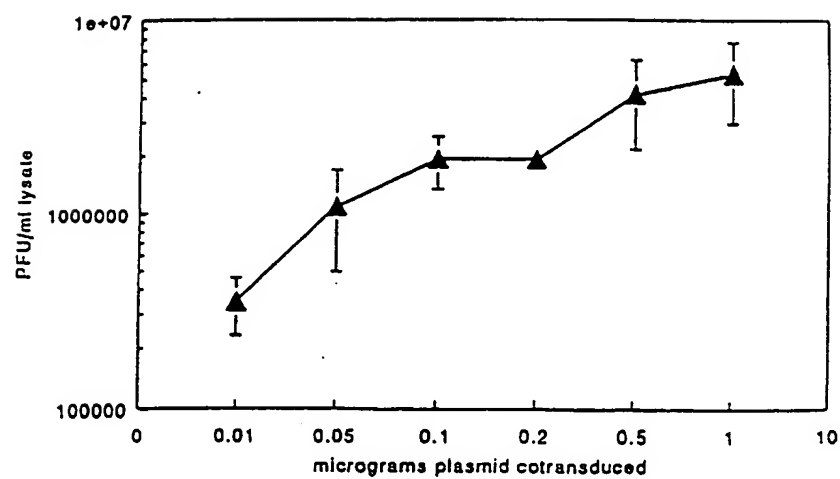
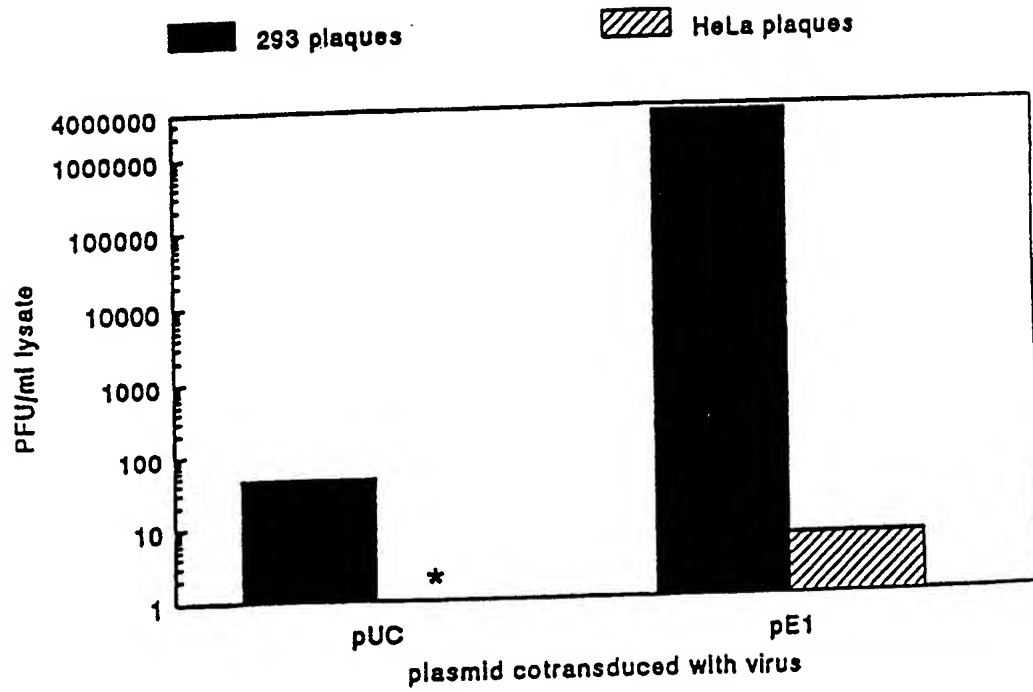


FIGURE 6b

8/15

**FIGURE 7**

SUBSTITUTE SHEET (RULE 26)

9/15

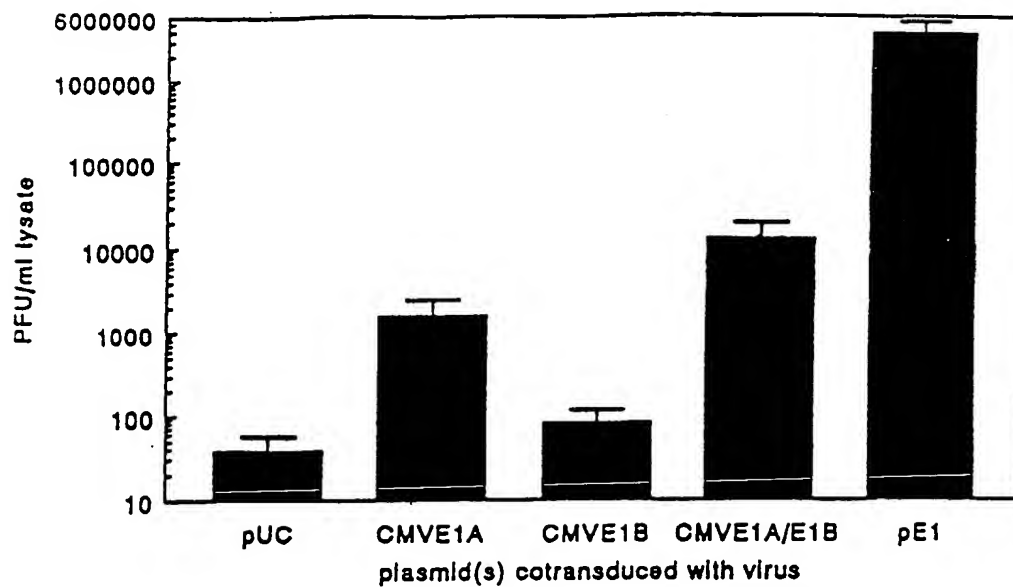


FIGURE 8

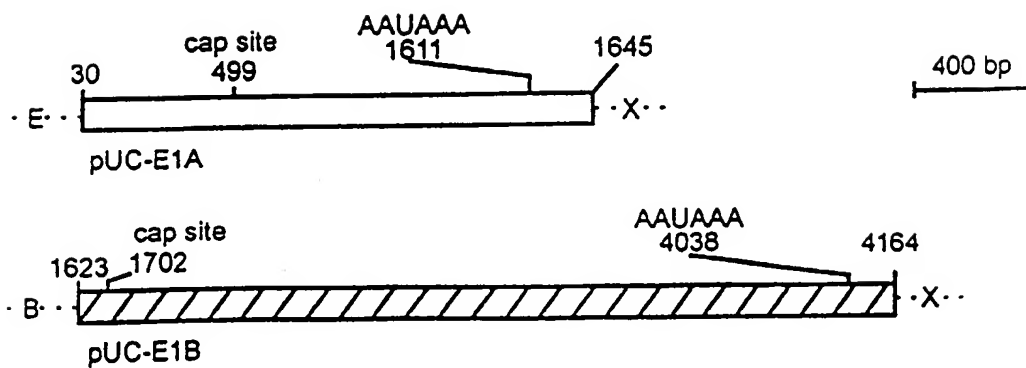


FIGURE 9

SUBSTITUTE SHEET (RULE 26)

10/15

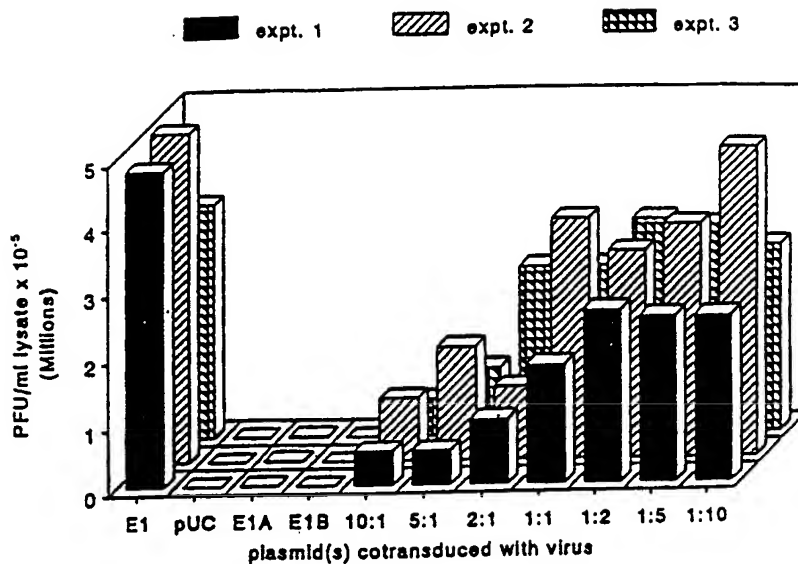


FIGURE 10

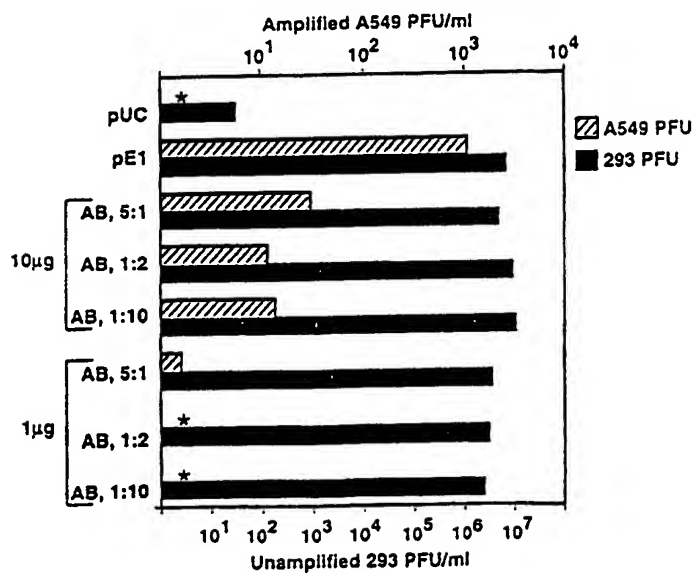


FIGURE 11

11/15

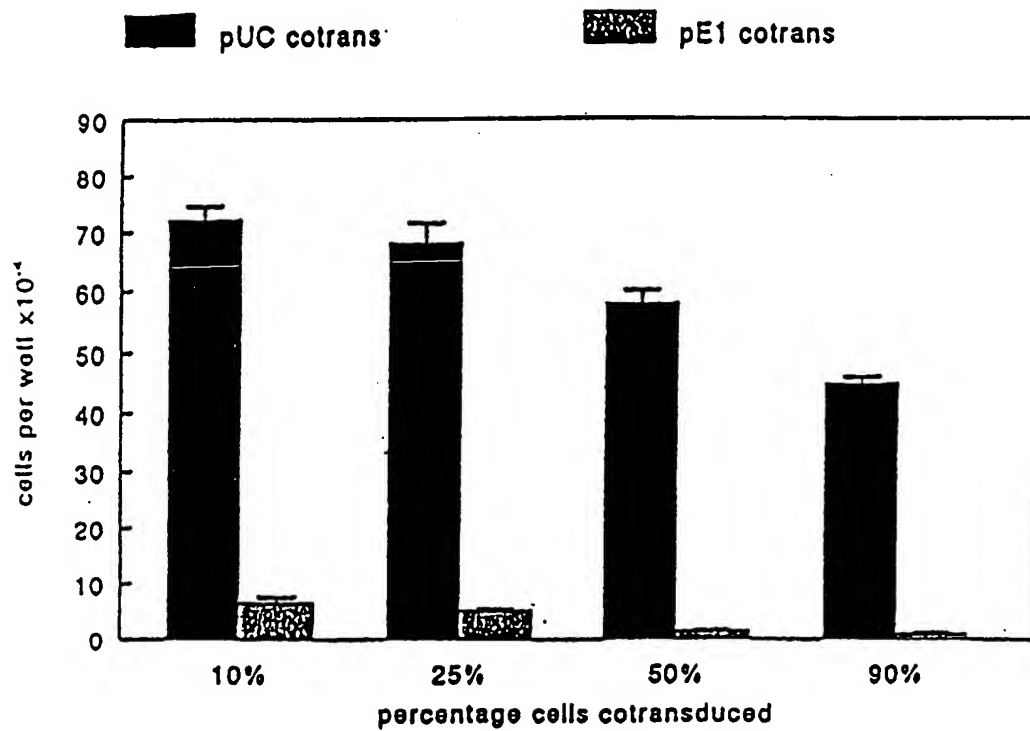


FIGURE 12

12/15

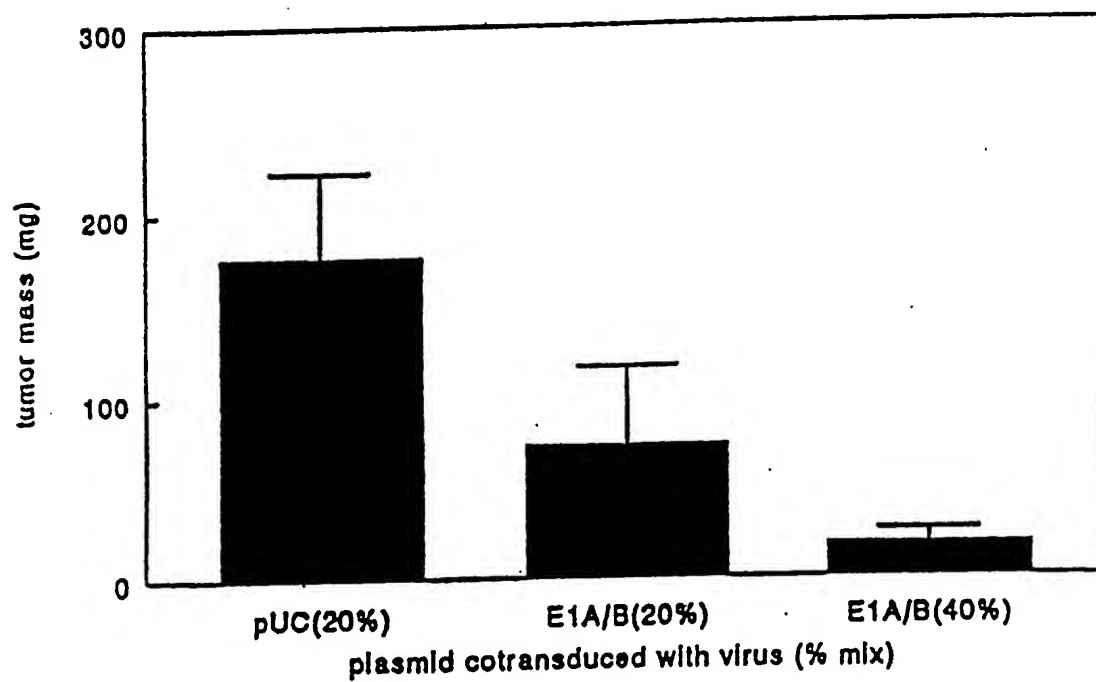


FIGURE 13

13/15

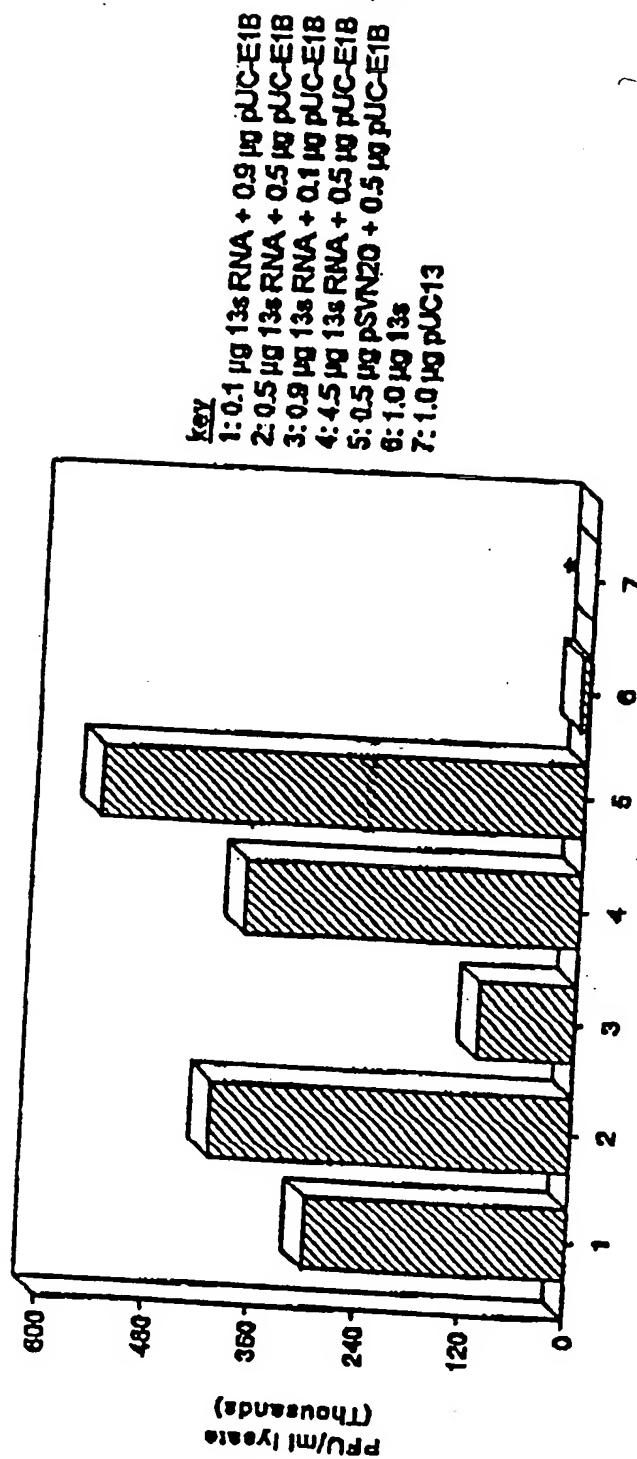
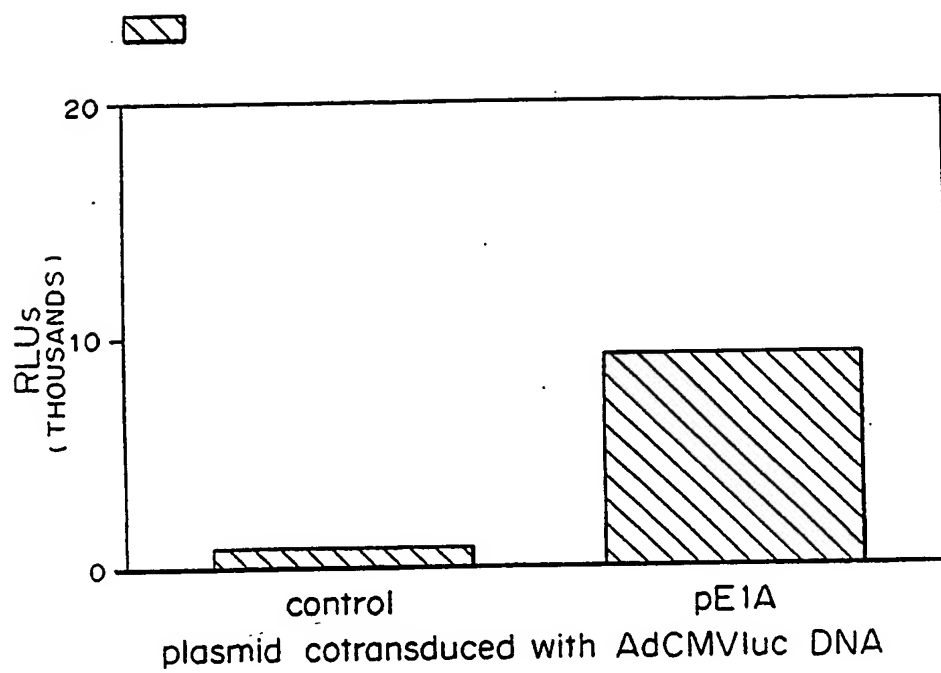
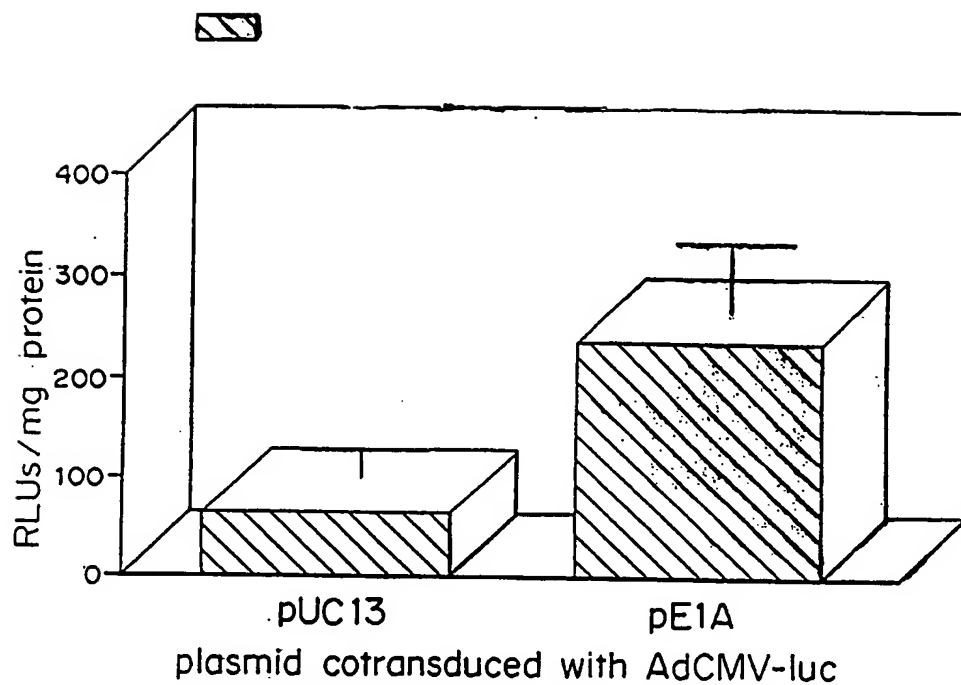


FIGURE 14

14/15

**FIGURE 15**

15/15

**FIGURE 16**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08885

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 63/00; A61K 48/00; C12N 5/00, 15/00

US CL : 424/93.2; 435/172.3, 240.2, 320.1; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2; 435/172.3, 240.2, 320.1; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FISHER et al. Biochemical and functional analysis of an adenovirus-based ligand complex for gene transfer. Biochemical Journal. 1994, Vol. 299, pages 49-58, see the entire document.	2, 4-7, 13, 15, 16, 19, 21
X	US 5,173,414 A (LEBKOWSKI et al.) 22 December 1992, see the entire document.	1, 8-12, 17, 18, 22
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Y		2, 4-7, 13, 15, 16, 19, 21

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 22 AUGUST 1996	Date of mailing of the international search report 16 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRUCE CAMPELL Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08885

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	MARKOWITZ et al. A Safe Packaging Line for Gene Transfer: Separating Viral Genes on Two Different Plasmids. Journal of Virology. April 1988, Vol, 62, No. 4, pages 1120-1124, see the entire document.	1, 8-12, 17, 18, 22 _____ 2, 4-7, 13, 15, 16, 19, 21
X — Y	QUANTIN et al. Adenovirus as an expression vector in muscle cells in vivo. Proceedings National Academy of Sciences USA. April 1992, Vol. 89, pages 2581-2584, see the entire document.	11, 12, 17 _____ 1, 2, 4-10, 13, 15, 16, 18, 19, 21, 22
Y	BREAKEFIELD et al. Herpes Simplex Virus for Gene Delivery to Neurons. The New Biologist. March 1991, Vol. 3; No. 3, pages 203-218, see the entire document.	1, 2, 4-13, 15-19, 21, 22
X — Y	GOLDSMITH et al. Trans Complementation of an E1A-Deleted Adenovirus with Codelivered E1A Sequences to Make Recombinant Adenoviral Producer Cells. Human Gene Therapy. November 1994, Vol. 5, pages 1341-1348, see the entire document.	1, 2, 4-6, 8-13, 15-19, 21, 22 _____ 7, 23

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